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MESOTHELIOMAS OF THE GENITAL TRACT

A report of five new cases and a survey of the literature.

By *Carl-Martin Fajers.*

(Received for publication July 3rd, 1948.)

The following report deals with a special type of benign tumours in the genital tract of men and women. In the literature they have been described as lymphangiomas, mesotheliomas, adenomatoid tumours, adenomas, adenocarcinomas, but they seem to represent a tumour specific for the genital tract. Up to 1942 18 male and 19 female cases have been published. In 1942 Masson, Riopelle, and Simard described 8 new cases, 3 of which were women, classifying them as mesotheliomas. In 1943 Evans published 15 cases including 2 females. In 1945 Golden and Ash's report appeared with 15 cases of their own, 2 of which were women. Since the authors could not arrive at any definite conclusion as to the genesis of the tumours they called them adenomatoid tumours. Ten*) isolated male cases have been published since 1942. The present material comprises 5 male cases.

A description is given of these 5 cases, followed by a general review, based on the author's experience and on the cases reported in the literature. Sixty-four*) tumours in men and 25 in women are described. Of these 13 male and 2 female cases have been tabulated by Evans; 13 male and 2 female cases are described by Golden and Ash. The remaining male cases and all known female ones are presented in table 1.

Report of cases.

Case 1. (91/1909) (The Surgical Department of the Kalmar Hospital and the Surgical Department of Lund Hospital). Man, age 27. As a child the patient had had measles and mumps without complications. He denied venereal disease. The anamnesis mentioned a trauma in the scrotal region in 1903. In June, 1908, the patient noticed a sudden hard and painful swelling in the

*) See end of this report for another case.

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
MALES				
1	Conforti 1910	23	A tumour for 5 months in the left epididymis (6,5 × 3,0 cm). No pain. Hydrocele.	Diffus and alveolar lymphangioendothelioma
2	Lardennois and Legène 1911	33	A tumour for a long time in the dorsal-side of the right testicle. 1 cm. in diameter.	Fibroma
3	Naegeli 1912	59	Incidental autopsy finding. Right testicle in the transition between the epididymis and the vas deferens.	»Misch tumor«
4	Sakaguchi 1916	32	Epididymis; 2 × 1,5 cm.	Adenomyoma
5	Stout 1917	57	Trauma against the scrotum at 12 years of age. Tumour in the lower part of the left epididymis; 1 cm. in diameter.	Fibromyoma
6	Hinman and Gibson 1924	78	A big scrotal tumour for about 11 months in the left epididymis.	
7	Masson 1924 (according to Nicod)		Spermatic cord.	
8	Rigano-Irrera 1925	47	A walnut-sized tumour for 6 months in the left testicle. In the beginning pain. Lues 4 years ago. Now WR and Meinelcke neg.	Lymphangioma simplex
9	Rubaschow 1927	23	A cystic tumour for 5 years in the lower part of the left spermatic cord; 2—3 times as big as the testicle.	Lymphangioma
10	Felsenreich 1929	38	Pain in the right inguinal region for 1 year. 4 cm. from the epididymis along the spermatic cord a 8 cm. long tumour.	Tumour from the Wolffian duct. Perhaps a carcinoma solidum simplex

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
11	Marcandier and Thomas 1930	41	Epididymis; walnut-sized.	Lymphangioma
12	Fischer, W. 1932	60	A little growing nodule for 6 years in the cauda of the left epididymis; 1,6 cm. in diameter.	Myo-adenofibroma
13	Mark, A. 1935 (according to Golden and Ash)			
14	Thompson 1936 case 38	29	Pain from the region of the testicle. Lower part of the right epididymis; a little tumour.	Adenocarcinoma, grade I
15	Thompson 1936 case 39	54	Trauma against the right testicle 4 years ago. Since that time a little nodule. Lower part of the right epididymis; a little nodule, 1 cm. in diameter.	Adenocarcinoma, grade II
16	Scalfi, A. 1936 (Scalfi, A. 1935)	44	Since some years a tumour. Lower part of the left testicle growing on the spermatic cord; walnut-sized. No pain.	Lymphangioma simplex
17	Falconer 1939	72	Incidental autopsy finding.	Adenofibromyoma
18	Nakamura 1939	37	A tumour in the right testicle for 17 years. No pain. Right testis; $2-3 \times 1,5 \times 0,7$ cm.	Lymphangioma simplex and cavernosum
19	Masson, Riopelle and Simard 1942 case 4	23	Spermatic cord; walnut-sized.	Mesothelioma
20	Masson, Riopelle and Simard 1942 case 5	31	Pain in the left groin. A tumour in the cauda of the left epididymis for 18 months; $2 \times 1,5$ cm.	Mesothelioma

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
21	Masson, Riopelle and Simard 1942 case 6	29	Pain in the left testicle for 4-5 days. No venereal diseases. Cauda of the left epididymis; pea-sized.	Mesothelioma
22	Masson, Riopelle and Simard 1942 case 7		A tumour in the right testicle for 2 years. Pain. Right epididymis; nut-sized.	Mesothelioma
23	Masson, Riopelle and Simard 1942 case 8	31	Operation on diagnosis ectopia testis sin. Albuginea on the dorsal-side of the left testicle; 1 mm in diameter.	Mesothelioma
24	Malisoff and Helpert 1943	57	A nodule in the right testicle for 17 years. Pain. Hydrocele. Caput of the right epididymis; 1,8 cm. in diam.	Mixed leiomyoma and lymphangioma
25	Robinson, J. N. 1945	30	A tumour in the lower part of the left epididymis for 5 years.	Most likely a mesothelioma
26	Robinson, J. N.	28	Pain and swelling 4 years ago in the left part of the scrotum. Tumour in the lower part of the left epididymis.	Most likely a mesothelioma
27	Bothe, Cristol and Devers 1946	42	13 years ago trauma against the scrotum. Since then swelling and a nodule. No pain. Nodular tumour in the lower part of the left epididymis; 4 cm. in diameter.	
28	Codnere and Flynn 1946 case 1	26	A tumour in the caput major epididymis sin. (1,8 × 1,2 cm.) for 10 months. No trauma. No pain. No venereal diseases.	»Adenomatoid tumor
29	Codnere and Flynn 1946 case 2	33	A tumour in the upper part of the right epididymis (1,2 × 0,3 cm.) for 7 years. No pain. Kahn neg.	»Adenomatoid tumor

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
30	Codnere and Flynn 1946 case 3	47	Hernia inguinalis dx. for 6 months. No trauma. No pain. No venereal diseases. Kohn neg. Bilat. hydrocele. Caput minor epididymis; $0,8 \times 0,6 \times 0,5$ cm.	»Adenomatoid tumor
31	Beneventi, F. 1947	38	A tumour in the globus minor epididymis sin. ($2 \times 2 \times 1,5$ cm.) for 4 months. Trauma 25 months ago. No pain. Now WR pos. but 2 years ago neg.	Perhaps a lymph-angioma
32	Falconer, B. 1947 case 1	39	A nut-sized tumour in the cauda of the right epididymis for 5 months.	Adenofibromyoma
33*	Falconer, B. 1947 case 2	36	A nut-sized scrotal tumour for 6 years.	Adenofibromyoma

CASES OF THE AUTHOR

34	Fajers, C.-M. 1948 case 1	27	Trauma against the scrotum 6 years ago. No pain. Cauda of the right epididymis; pea-sized.	Mesothelioma
35	Fajers, C.-M. 1948 case 2	35	Nothing of interest. No trauma. A tumour for 2—3 months. Pain. Left epididymis; pea-sized.	Mesothelioma
36	Fajers, C.-M. 1948 case 3	50	Tumour for 1 year. No pain. Cauda of the left epididymis; double pea-sized.	Mesothelioma
37	Fajers, C.-M. 1948 case 4	45	Piles. Tumour for 5 years. No pain. Cauda of the right epididymis; bean-sized.	Mesothelioma
38	Fajers, C.-M. 1948 case 5	58	Nothing of interest, no trauma. A tumour for about 2 years. Some pain. Lowest part of the right testicle; walnut-sized.	Mesothelioma

*) See end of this report for case 33½.

	Author and date	Age	Anamnesis, symptoms, loca- lization and size	Diagnosis
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F E M A L E S

39	Höhne 1901		Uterine fibroids. In the upper right wall of the tube, 1 cm. lateral of the corner of the tube; cherry-sized.	Lymphangioma
40	Dienst 1905		Uterine fibroids. In the ampullary part of the tube; pea-sized nodule.	Myxofibrotic capillary angioma
41	Kermauner 1907	50	Married 2-para. An abdominal tumour for 5 months. Irregular uterine bleedings for 1 years. Uterine fibroids. In the isthmic extremity of the tube a bean-sized thickening.	Lymphangioma with some malignancy
42	Franz and Leighton 1910 and 1912	53	Nullipara. Menopause 4 years ago. Irregular uterine bleedings for 1 year. Adenocarcinoma uteri, metroendometritis purulenta; myoma corporis et isthmi uteri. In the ampullary part of the left tube; cherry-sized.	Lymphangioma
43	Frankl 1912	38	Married. Menorrhagia and metrorrhagia. Uterine fibroids. In the middle of the left tube; pea-sized tumour.	Lymphangioma
44	Küster 1914		Cancer uteri. On the tube; walnut-sized tumour.	Partly cystic lymphangioma
45	Dietrich 1923	30	2-para. Retroflexion of the uterus. No myomas. Pelvic pain for 8 years. In the isthmic part of the right tube; nut-sized tumour.	Lymphangioma
46	Strong 1924 case 1	48	Married. Abdominal tumour. Fibrosis uteri. Fibroma ovarii. No myoma. 1 cm. from the isthmic extremity of the tube a tumour, 5 mm in diam.	Lymphangioma

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
47	Strong 1924 case 2	52	Married. Pelvic pain and haemorrhage. No uterine fibroids. Inflammatory condition of the uterus. In the wall of one of the tubes; 5 mm in diameter.	Lymphangioma
48	Aschheim 1925 case 1		Myoma uteri and fibroma ovarii. In the ampullary part of the tube; pea-sized subserous tumour.	Lymphangioma
49	Aschheim 1925 case 2		Uterine fibroids. In the isthmic part of the tube; nut-sized tumour.	Lymphangioma
50	Aschheim 1925 case 5	40	Married multipara. Abdominal pain for 14 days. In the tube; child's head-sized tumour.	Lymphangioma of the tube, partly solid partly cystic
51	Todoroff 1928			
52	Silva 1928	86	Married. Incidental autopsy finding. Little, atrophic uterine body. At the isthmic part of the left tube a tumour 6 × 4 mm.	
53	Schiffman 1929	74	Married. Pelvic haemorrhage. Ca. ovarii. 5 cm. from the uterine extremity of the tube a cherry-sized tumour.	Lymphangioma
54	Nicod 1934	77	Autopsy. The right tube and ovary form a child-head-sized cystic tumour. »Kyste wolffien multiloculaire«. Some uterine fibroids. In the left tube; nut-sized firm tumour.	»Réticulomes ou réticulo-endothéliomes lymphatiques«
55	v. Szathmary 1937	53	Nullipara. Since four years irregular uterine bleedings. Fist-sized uterine fibroid. In the middle of the 9 cm. long left tube; a firm beansized nodule.	Lymphangioma

	Author and date	Age	Anamnesis, symptoms, localization and size	Diagnosis
56	Sanes and Warner 1939	55	Married unipara. Abdominal tumour for 18 months. Pain. Menses earlier normal; for 1½ year irregular menorrhagia. Uterine fibroids. 8 cm. from the uterine extremity of the left tube a tumour 1.1 cm. in diameter.	Lymphangioma with distinct endothelial proliferation
57	Masson, Riopelle and Simard 1942 case 1	25	Married nullipara. Abdominal pain in the right fossa iliaca for 1 year. Endometriosis ovarii. No uterine fibroids. On the dorsal surface of the uterus a tumour, 1 cm. in diameter.	Mesothelioma
58	Masson, Riopelle and Simard 1942 case 2	50	Multipara. No pain. Menorrhagia for 6 months. Endometrial hyperplasi. No uterine fibroids. Endosalpingitis tuberculosa. On the ventral surface of the uterine body at the attachment of the round ligament a nut-sized tumour.	Mesothelioma
59	Masson, Riopelle and Simard 1942 case 3	39	Multipara. Peritonitis 9 years ago. »Ovaires scléro-kystiques«. A badly delimited fibromyoma 2,5—3 cm. in diameter.	Mesothelioma
60	Evans 1943	52	Pelvic symptoms. In the uterine body a rounded intramural tumour mass about 7 cm. in diameter.	Mesothelioma
61	Evans 1943	45	Profuse menstruation for 3 years. Uterine fibroids. On one of the tubes; a small rounded tumour 8 mm. in diameter.	Mesothelioma
62	Golden and Ash 1945	67	Incidental autopsy finding. Uterine tube.	»Adenomatoid tumo
63	Golden and Ash 1945		Incidental finding at operation »Encapsulated tumour on the Falloppian tube«.	»Adenomatoid tumo

right groin. He received hospital treatment for 14 days under the diagnosis of lymphadenitis of the right groin. In October 1908 there was a sudden swelling and later on pain in the right testicle.

On examination in February, 1909, the right testicle was the size of a hen's egg, softer than normal and very painful on pressure. The right epididymis was considerably enlarged, hard, firm, and uneven, enclosing the testicle like a hook. On the ventral side of the testicle there was an area with suspicious fluctuation. The left testicle was softer and smaller than normal. Otherwise nothing noteworthy. On the diagnosis of tuberculous epididymitis the right testis was removed. The epididymis was entirely liquefied and in the anterior part of the testicle a clear liquid was found in a delimited area. In the lower part of the right epididymis there was a peasized tumour. The healing was uneventful. After operation the patient experienced only slight discomfort from the inguinal region. (Information by letter in December 1947). The tumour was classified histologically as a lymphangioma.

Case 2. (2805/1935) (Kullberg Hospital, Katrineholm). Man, age 35. For 2—3 months the patient had had a small tumour in the left epididymis, which had grown slightly and lately had become painful.

The clinical examination showed a firm, painless tumour somewhat larger than a pea in the left epididymis. After the operation the tumour measured $1.5 \times 1.0 \times 0.5$ cm; it was firm, elastic, yellowish white in color, and encapsulated. The recovery was uneventful. The patient wrote in December 1947 that he had not experienced any discomfort from the inguinal region. The histological diagnosis was »benign tumour, probably an angioma«.

Case 3. (885/1937) (Norrköping City Hospital). Man, age 50. The patient was operated upon for stomach ulcer in 1922 and had not noticed any trouble from the stomach since then. For a year he had had a slowly growing, painless tumour in the lower part of the left testicle.

The clinical examination revealed a rounded, fairly hard, painless, well delimited tumour, double the size of a pea, in the lower part of the left epididymis. On operation a well encapsulated, firm, elastic, greyish white tumour was removed which measured 1 cm. in diameter. Uneventful healing. The post-operative course presented no discomfort from this region. (Information by letter in December 1947). — The pathological-anatomical diagnosis was a tumour without any sign of malignancy, probably an angioma.

Case 4. (7260/1944) (The Surgical Department, Lund). Man, age 45. For 5 years the patient had had a firm, painless, peasized tumour in the lower part of the left testicle. Only neurastenic symptoms.

Epididymectomy on the right side was performed and a beansized tumour was removed from the fibrotic cauda epididymis. A small hydrocele was present. Uneventful healing. In a letter dated December 1947, the patient wrote that he experienced tension in the groin due to the fact that »the testicle had not descended into position after the operation«. The tumour was 1 cm. in diameter, firm, elastic, yellowish white, well encapsulated, and was interpreted histologically as an angioma.

Case 5. (1973/1945) (The Surgical Department, Hälsingborg). Man, age 58. The patient could not recall trauma in the serotal region. For 2 years he had a tumour in the right half of the serotum which had lately become painful.

On operation a walnut-sized tumour was removed from the lower part.

of the right testicle. The tumour was white, firm, elastic, and encapsulated. It showed the histological picture of an angioma, probably lymphangioma. Uneventful healing. There was no further discomfort from this region. (Information by letter in December, 1947).

Microscopic examination¹):

All 5 tumours were strikingly similar in structure. In a framework of collagenous connective tissue and smooth muscle fibres there were epithelium-like cells in solid cords or channels, sometimes arranged in acinar formations with a central lumen. The cells were flat to cuboidal or low-columnar, in some places spider-like with vacuolated cytoplasm. Many of the cells showed a brush-border (fig. 5) and sometimes a central flagellum. This brush-border was most evident on the flat cells. The central flagellum rose somewhat above the brush-border. Some cells had a signet-ring-like appearance with eccentric nuclei. The nuclei were rounded to ovoid, sometimes kidney-like with a granular to filamentous chromatin and often evident nucleoli. Very few mitoses



Fig. 1.

Case 2: The tumours have an angiomatous appearance with reticular fibrils forming a basal membrane next to the gland-like structures. $\times 90$.

(1) Stainings: Hematoxylin-eosin; hematoxylin-v. Gieson; elastic tissue staining according to Fraenkel; mucicarmine; azocarmine according to Mallory; reticulate fibril staining according to Pap and Scharlach-R.

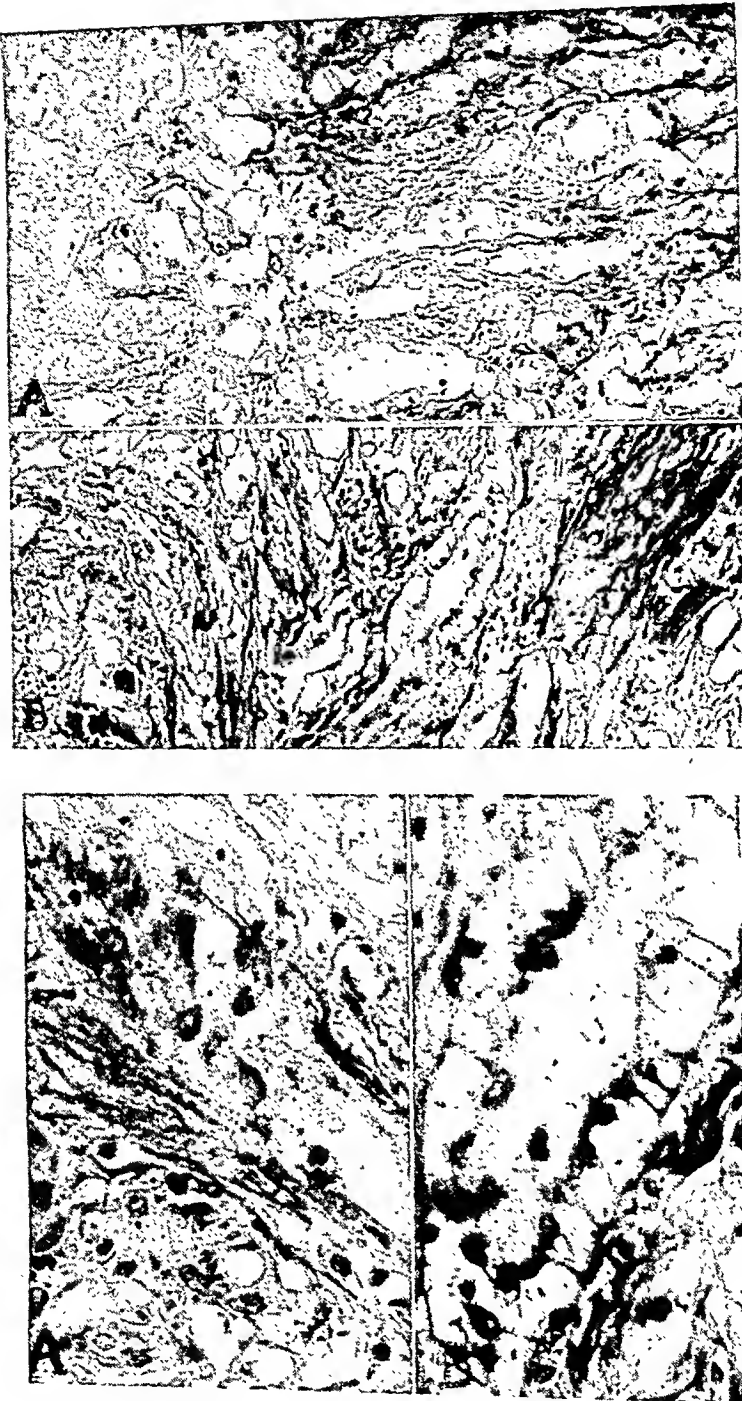


Fig. 2 and 3.

- A. Case 2: Parts of the tumours are more solid with gland-like spaces surrounded by cuboidal to low-columnar epithelium-like cells with a vacuolated cytoplasm and rounded or ovoid nucleus. $\times 150$ and 450 .
- B. Case 3: Other parts show signet-ring-like cells with a highly vacuolated cytoplasm and a flat to ovoid nucleus. $\times 150$ and 450 .

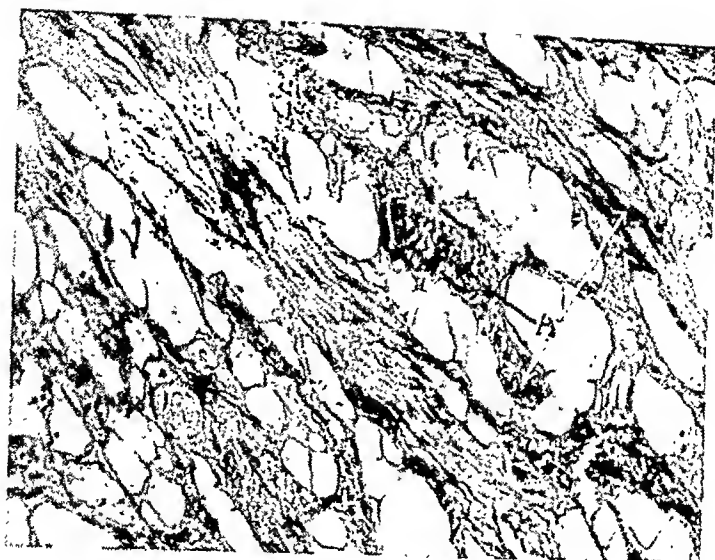


Fig. 4.

Case 4: Smooth muscle bundles are seen in the collagenous connective tissue at A. $\times 250$.

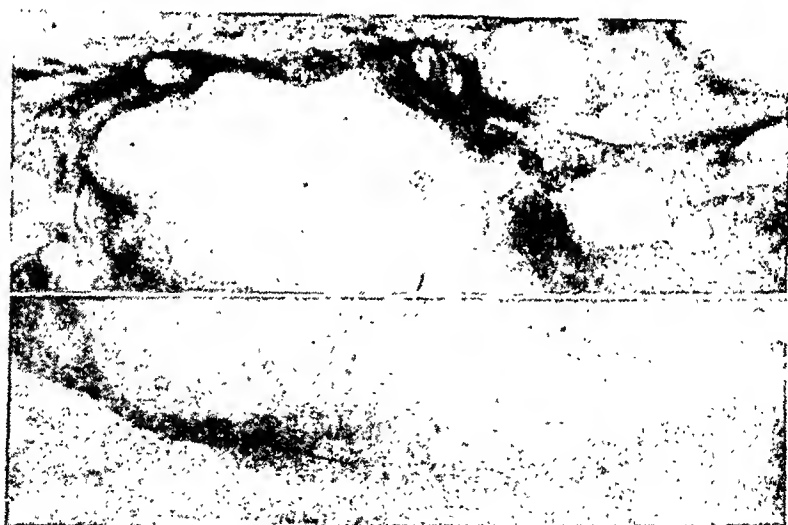


Fig. 5.

Case: Mainly the flat epithelium-like cells lining the gland-like spaces show a brush-border, A $\times 750$; B $\times 1300$.

were observed. The signet-ring-like cells seemed to serve as the origin of new acinar formations. In some places the gland-like formations were seen to divide in branches. The epithelium-like cells showed everywhere a light, foamy cytoplasm (fig. 2 and fig. 3). Staining for fat was negative. Haemorrhages or pigments were not observed. There were no giant cells. The contents of the channels and the cytoplasm were staining faintly rose-coloured with mucicarmine, except in case 1.

In the lumen of the gland-like structures mononucleated cells were present, which seemed to be desquamated from the epithelium-like cells.

The smooth muscle fibres appeared mainly in the periphery of the tumours (fig. 4). In case 5, the fibres seemed to lie in two perpendicular layers at right angles to each other. The collagenous connective-tissue was most abundant in the centre of the tumour, wholly dominating the smooth muscle fibres. In the periphery, however, the collagenous connective tissue was sparse whereas the smooth musculature was more abundant. Next to the gland-like structures reticular fibrils were seen, forming a basal membrane, and outside the fibrils the collagenous fibres appeared (fig. 1). In the utmost periphery a more or less pronounced capsule of collagenous connective tissue was found in all the cases. On the surface of this capsule mesothelial cells were seen in case 2 in a limited area of the vaginal cavity. The cells dipped into the capsule, but no connection between the cells and the gland-like structures of the tumour were seen anywhere, nor did these mesothelial cells appear to create acinar structures.

A diffuse infiltration of round cells was present in the tumours, most abundant in the periphery where they even formed groups of lymphocytes (most pronounced in case 5). Eosinophilic leucocytes occurred sparingly in case 2 and 4 but were otherwise lacking. There were no neutrophile leucocytes present and only a few plasma cells. In the aggregations of lymphoid cells there appeared a network of reticular fibrils.

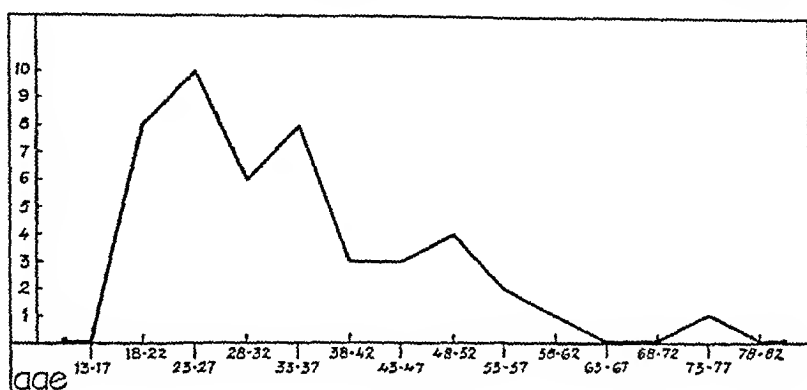
The tumours may thus be classified as follows: adenomatoid tumours, with gland-like structures of epithelium-like cells with brush-border, with interstitial tissue framework of smooth muscle tissue, collagenous connective-tissue and an infiltration of round cells, which form rounded groups, especially in the periphery. A sparse network of vessels is observed in the tumours.

General survey.

Occurrence: Sixty-four of the 89 tumours were found in men and 25 in women. They seem thus to be more common in men. It is very likely, however, that the number for women is too low, partly because the tumours are too small to give clinical symptoms, and partly because they are overlooked in the autopsy on account of their insignificant size. They are perhaps allowed to pass as myomas without microscopic examination.

Age: The age is mentioned in 60 male cases. The tumours however, have existed long before they were removed. The operation often took place in connection with military service which may explain the fact that the younger ages are more represented than the older. In some

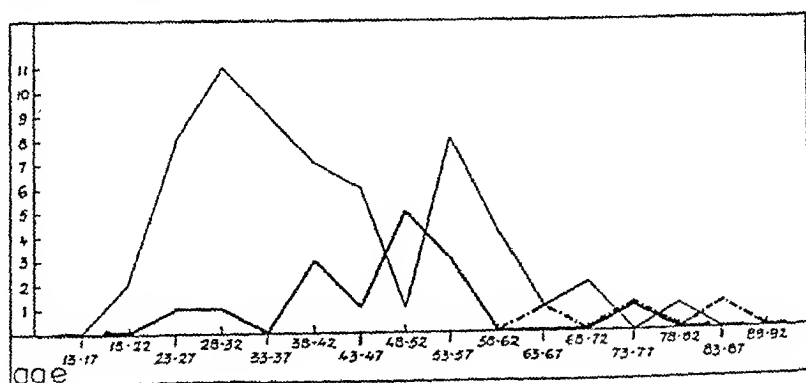
cases the tumours were accidental secondary findings. The age of interest is that at which the tumours first appeared. It is mentioned in 46 cases (see Curve 1). The tumours seem to be exceedingly rare before puberty. (There is only one case with an anamnesis starting before the age of 20 and Edward and Blumer's case in which the tumour was said to have been present since the early teens). Maximal frequency occurs in the ages between 20 and 40. The tumours seem to become less frequent with increasing age. This fact however may be due to a reduction in the age classes and to a possible selection.



Curve 1.

Age in men when the tumour first appeared. (46 cases).

The age is mentioned in 18 female cases (see Curve 2). Three of these are autopsy findings. The curve shows a peak, although insignificant, at the menopause. This is explained by the fact that the tumours have always (with the exception of case 50) been secondary findings in operations for complaints from the internal genitalia.



Curve 2.

Age when the tumour was removed;

- female cases.
- male cases.
- female autopsy findings.

Localization: The site of the tumours in men is mentioned in 44 case (see fig. 6):

- 4 were found in close relation to the caput epididymis.
- 1 between the caput epididymis and the spermatic cord.
- 28 in the cauda epididymis.
- 1 in the transition between the epididymis and the vas deferens.
- 6 intimately related to the lower pole of the testicle.
- 4 long the spermatic cord.

Finally, in a few cases, they have been firmly adherent to the tunica vaginalis or have appeared to originate from the tunica albuginea.

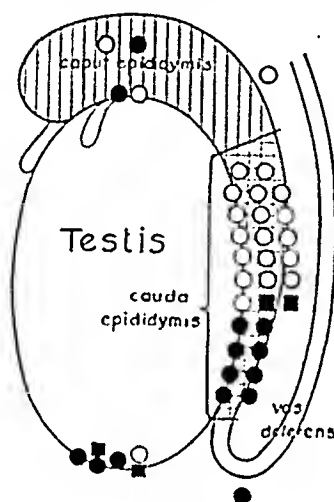


Fig. 6.

Diagrammatic representation of the localization of the male tumours.

- side not mentioned.
- on the left side.
- on the right side.

It is evident from the schematic diagram that in the majority of cases the tumours have been localized to the cauda epididymis or its immediate vicinity; this is valid for 35 cases out of 44. — In 29 cases the tumours were found on the left side, in 20 on the right, and in 15 cases the side was not mentioned.

The side of the tumours in women is mentioned in 25 cases. The distribution appears from Fig. 7.

19 on or in the Fallopian tube:

- 6 in the isthmie part of the Fallopian tube.
- 4 in the middle part of the Fallopian tube.
- 3 in the ampullary part of the Fallopian tube.
- 6 on the tube, not clearly specified.

5 on the surface of the uterus.

1 case, localization unknown, as the original report was unobtainable.

Six of the tumours were situated to the left and 3 to the right.

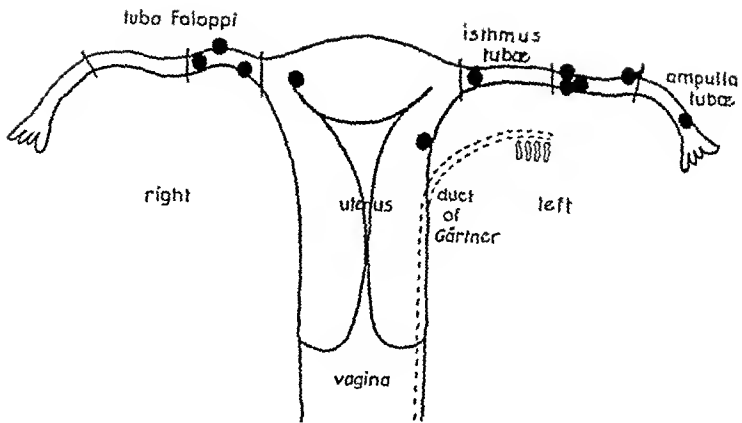


Fig. 7.

Diagrammatic representation of the localization of the female tumours.
Exact localization not mentioned in 14 cases.

Macroscopic appearance: The tumours in both men and women are usually small, about the size of a hazel-nut. In a few tumours cystic structures are found but these were small except in case 50 where the tumour could be palpated. It was as large as a child's head and consisted of one cystic and one solid area. The tumours were all unilateral.

They are as a rule rounded to ovoid in shape, but appear sometimes as flattened discs. This was especially the case in women where the tumour had grown on the uterus, and in the male cases 1, 6 and 10 in the table.

The tumours are macroscopically well delimited and in most cases encapsulated. They are generally firm and elastic.

The colour as a rule is greyish-white to greyish-yellow without any signs of haemorrhages and with a somewhat darker shade in the periphery in those cases where a capsule was present.

Symptomatology: The tumours as a rule have been without symptoms in both men and women.

Hydrocele is mentioned in 9 cases. The exudation is probably caused by the irritation incited by the tumour through its very presence in the vaginal cavity — it would be justifiable to expect a greater frequency of this symptom.

The tumour was combined with pain in 16 male cases. In 5 cases of hydrocele there was no pain and the combination of hydrocele and pain was found in only 3 cases. This indicates that in most cases hydrocele is not the cause of pain.

Pain was mentioned in a few female cases but was probably not related to the occurrence of these tumours.

Prognosis: The prognosis is always very good. The tumours are benign and no recurrences are reported.

Pathogenesis.

Trauma: In only 5 or perhaps 6 male cases the anamnesis mentioned trauma in the scrotal or inguinal region. It is possible that the trauma increased the growth of the tumour in these cases. However, no causative rôle can be attributed to the trauma. — No traumas were reported in women.

Veneral diseases: Only 2 men have admitted venereal infection. The number is probably too low, but there is no reason to assume any relation between venereal disease and the occurrence of the tumour. — Venereal diseases are not reported for women.

Inflammation: The tumours showed a sparse, diffuse infiltration of round cells, chiefly lymphocytes, sometimes aggregated in groups, mostly in the periphery of the tumours. They may be of secondary nature elicited by the growth. The organoid structure of the tumours (e. g., their delimited character) speaks against an inflammatory genesis.

Endocrine origin: Falconer has suggested that the tumours probably originate from the Wolffian duct and transverse ducts of the primordial kidney and that their development may be hormonally determined. However, no endocrine disturbances were mentioned in the male cases. Of the female cases myoma of the uterus was present in 13 cases and was denied in 6. Two cases had carcinoma of the uterus, 1 carcinoma of the ovary, 3 inflammatory processes in the uterus, 1 endometrosis of the ovaries, 1 ovarian cysts and 1 ovarian fibroma. As the tumours were accidental findings during the operations, their co-existence with these conditions does not mean causal connexion. Moreover, there are no proofs of an endocrine genesis of any of these diseases, perhaps with the exception of the myomas. In all the female cases anamnesis shows irregular menstruation.

The number of pregnancies (nullipara 3, multipara 5) is not significant for the occurrence of the tumour.

Histogenesis.

Angiomatous character: Several cases have been diagnosed as angiomias. For many reasons, however, this diagnosis can be rejected. The tumour cells differed from the endothelial cells which line the vascular spaces of the angiomias, in being cuboidal to low-columnar, whereas the latter always retain their typical structure with bulging nuclei in spindle-shaped cells. Moreover, the tumour cells showed a mucous secretion, and in the author's cases as well as Masson's cilia, which are never seen in angiomias.

Epithelial character: The tumours have been diagnosed as *adenocarcinomas* (Thompson, Hinman & Gibson, and others), but at the same time it has been stressed that the tumours probably were of he-

nign character. This is evidently true. Under the microscope the tumours appeared to be benign and the mitoses, if any, were very sparse and of normal appearance. Further, the tumours have existed in men for a long time without giving rise to metastases. No relapse is reported either.

Gordon-Taylor and Ommaney-Davys, Blummer and Edwards believed the tumours to be *adenomas*. Falconer denominated his tumours *adenofibromyomas*; Sakaguchi supposed his case to be an *adenomyoma*, and Fischer diagnosed his as a *myoadenofibroma*. Golden and Ash introduced the name of *adenomatoid tumours* and this diagnosis was adopted also by Codnere and Flynn. All these authors considered the possibility of the tumours having their origin in the Wolffian body (mesonephron) or in aberrant cells connected with the Wolffian duct.

• A genesis from mesonephron or aberrant cells connected with the

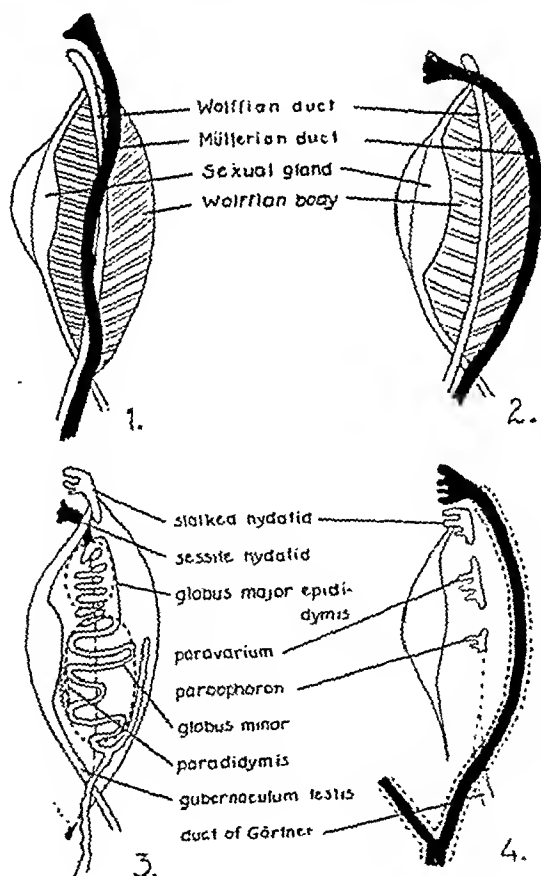


Fig. 8.

Diagrammatic representation of development of genitourinary tract. (Modified after Heister). Dotted line showing the localization of the tumours. Müllerian duct and derivatives are jet black. Wolffian body and derivatives include all other structures except sex glands. 1, indifferent type; 2, indifferent type, later stage; 3, male type; 4, female type.

Wolffian duct might explain the tumours in men but hardly those in women. The female tumours would then be found mainly along the remains of the Wolffian duct, that is in the epoophoron, the paroophoron, the ligamentum rotundum uteri, and Gärtner's and Skene's ducts. This is not the case, however (Fig. 8). The tumours were instead localized to places on the tube and the uterus where the Wolffian duct does not pass and where remnants of the duct are unexpected.

The Müllerian duct must also be considered when epithelium-like structures in these regions are concerned. These ducts develop from the coelom by folding. The anatomical localization of the tumours both in men and women agrees fairly well with this assumption. (Fig. 8). The tumours would then, however, in the females be expected to show cyclic changes similar to those of the female genital tract. This is not observed, however, which perhaps renders this genesis less probable.

In a report from 1947 Hartz mentioned the occurrence of Walther cell rests or Brenner-like epithelium in the serosa of the epididymis. However, no such rests are seen in the capsule of the tumours nor do the tumours show any histological similarities to the Brenner tumours.

Mesothelial character: An origin from the mesothelium was assumed by Masson et al. and by Evans. The mesothelium is believed to originate from the coelom epithelium which is very polyvalent. According to Maximow the coelom cells possess the power of creating mesodermal epithelium, smooth muscles, etc.

The mesodermal epithelium, the mesothelium, also has a great capacity of differentiation which the ordinary epithelium does not have. In tissue cultures it can form structures resembling uterine glands; it can develop into fibroblasts and so on. The mesothelial cells have brush-borders and they have the power of forming collagenous fibrils. They may form signet-ring-like cells with a content giving positive mucicarmine-reaction. Sometimes in inflammatory or irritated conditions it gives rise to giant cells, sometimes it is desquamated from the surrounding after which it forms mononuclear cells.

The mesothelial tumours, the nature of which, however, is very debatable, have a varied histological structure combining connective, epithelium-like and gland-like elements with the mentioned special capacities of the mesothelium. Cysts are often found in these tumours. The cases of Masson et al. all showed an intimate combination of connective tissue, epithelium-like cells, gland-like spaces and even smooth muscle bundles. In some of the tumours they found giant cells and in all there were signet-ring-like cells with mucicarmine positive contents. The acinar spaces contained mononuclear cells.

Further they found typical brush-borders in some of the tumours. The present author's five cases are, in all respects except for the absence of giant cells, identical with Mason's. When studying slides from one of Falconer's cases (no: 32 in table 1) a brush-border could be demonstrated there too.

These fact together with the localization of the tumours in or near the peritoneum or its derivatives (cavum vaginale testis) support the theory of mesothelioma.

Discussion.

It seems probable that the tumours have developed either from displaced cells (1) from the Wolffian duct or body, or (2) from the Müllerian duct, or (3) from mesothelial cells of the genital tract.

The first two theories are favored by the organoid structure of the tumours, the presence of smooth muscles and epithelium-like cells with a brush-border and in men also by the site of the tumours. The absence of any structures resembling primitive glomeruli and the localization of the female tumours, shown in fig. 8, speak against the first theory. As the tumours are identical in the two sexes a common origin seems to be most likely and thus this theory seems less probable. — The tumours in the females are localized on the abdominal surface of the uterine body. In the tubes the tumours seems to have been in, not on the walls in most cases, but the size of these small tumours makes an exact localization impossible. It is possible that the tubal tumours too have started from the abdominal surface as is the case in the uterine tumours. This and the fact that the tumours never show cyclic changes, endometriosis or any similarity to tumours never Müllerian duct speak against the Müllerian-duct-theory.

The mesothelial theory, on the other hand, is supported by the localization of the tumours on the serous membranes of the abdominal cavity, the presence of a brush-border, the appearance of the epithelium-like cells and their capacity of producing a mucicarmine-positive secretion, as well as by the tendency of cyst formation.

But how is the abundant occurrence of smooth muscle bundles in intimate combination with the other structures to be explained? The muscle cells may, as is held by Masson et al., originate from the surrounding tissue, as the effect of inflammatory irritation. This view is supported by the distribution of the muscle bundles, which appear more abundantly in the periphery. It is also conceivable that the muscles originate from the mesothelial cells themselves in analogy with the formation of muscles from the myoepithelium. There is, however, no information to this effect. No transition is seen between the smooth muscle cells and the mesothelial cells. In the myoepithelium the transition of the epithelial cells into smooth muscles can be followed. Myoepithelial tumours usually show an aggregation of epi-

thelial cells somewhere in the glandular acini where this transition can easily be demonstrated. No such aggregation of mesothelial cells was found in the cases reported here and the basal membranes were covered everywhere by a single layer of cells.

There is a third alternative. A group of mesothelial cells may have been isolated from the peritoneum in the embryonic stage and have been intimately mingled with the underlying musculature where they start growing later on. This can perhaps explain the intimate relation between collagenous connective tissue, gland-like formations and smooth muscle cells in the tumour.

One might argue against a supposed origin from the mesothelium that identical tumours are not reported from other parts of the peritoneal surface nor from the pleuras. However, the reported mesothelioma from the pleuro-peritoneum is of an heterogenous and debated nature and some of them show, in fact, structures, strongly resembling these tumours from the genital tract. It is also possible that the peritoneal mesothelium from the genital tract has specific potentialities.

Summary.

1. This report discusses a special type of benign tumours in the genital tract. Eighty-nine cases altogether are described in the literature, 64 of which are men. Thirty-eight of the male cases and all female ones are tabulated together with brief anamnestical data etc.

2. The tumours are exceedingly rare before puberty. In men the maximal frequency occurs in the age-group 20—40 years and in women at the menopause. The tumours are probably more frequent in women than appears from the number of cases.

3. In men about 80 % of the tumours are located in the epididymis region. A few tumours are found in the caput epididymis region and along the spermatic cord. In women most tumours occur on or in the tubes and a few on the external surface of the uteri.

4. The tumours are generally small, the size of hazel-nut; round to ovoid, well delimited and often encapsulated, greyish-white to greyish-yellow in colour, firmly elastic in consistency.

5. Microscopically the tumours are classified as being of adenomatoid type with gland-like structures of varying size and shape and mucicarmine-positive contents as well as an interstitial tissue framework of smooth muscle tissue and collagenous connective tissue. The epithelium-like cells from which gland-like structures arise are flat to low-columnar and provided with a brush-border and a central flagellum. In the periphery of the encapsulated tumours groups of round cells are present.

6. Hydrocele was present in a few male cases and pain in about

25 % of the cases. Pain was reported also in women but is scarcely caused by the tumours which almost always were purely secondary findings. Myoma of the uterus was present in 13 cases.

7. Trauma, venereal diseases, inflammation, and the number of gravidities have no causative significance for the appearance of the tumours.

8. The tumours are not angiomas. A genesis from the Müllerian duct or from the Wolffian duct or body seems less probable. In accordance with Masson an origin from the mesothelial cells of the peritoneum seems to be most likely.

9. The tumours are classified as mesotheliomas.

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*) Additional note at the proof-reading Case 33½: Patterson and Mogg: Brit. J. Surg. 1947, 34: 427—429. »A case of mesothelioma of the epididymis«. — Age not mentioned. Encapsulated tumour about 2.5 cm in each diam. in the globus major of the right epididymis for about one year. Trauma 6 years ago. No pain. No hydrocele.

STUDIES OF THE LIFE-CYCLE OF PROTEUS HAUSER

By Johs. Kvittingen.

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A microbe one meets with a good deal in the routine work of a bacteriological laboratory is *Proteus Hauser*. The reason why this microbe swarms and how it happens that on some plates it forms an even film and on others more or less regularly concentric zones, are questions which have long interested me.

In the Autumn of 1946, I made some studies of the »individuals« in a swarming proteus-culture, working out from the centre step by step to the edge. Smear-preparations were made, being stained after flame-fixation with carbolfuchsin, and it was seen that all the microbes in the preparation taken from the centre were short rods, whilst in that from the edge practically nothing but long rods were to be found. The test was then carried out again with different cultures and it was found that the picture from the centre was fairly constant, but that the preparations taken from the periphery contained in some cases long rods only; in other cases all stages from long to very short. It was, moreover, found that in some of the preparations from the edge, the rods were homogeneously stained, whilst in others they were packed with granules. The same variations in morphology and staining were found on several repetitions of the test and it could therefore be assumed that this was a case of real changes of the microbe and not of a technical error.

These findings, however, gave no explanation as to the mechanism of spreading and also a new question presented itself: Why was it that in one preparation the microbes were homogeneously stained and that in another taken, for instance, half an hour later, they were granulated?

A new method of making of preparations was then tried. 4—5 mm.

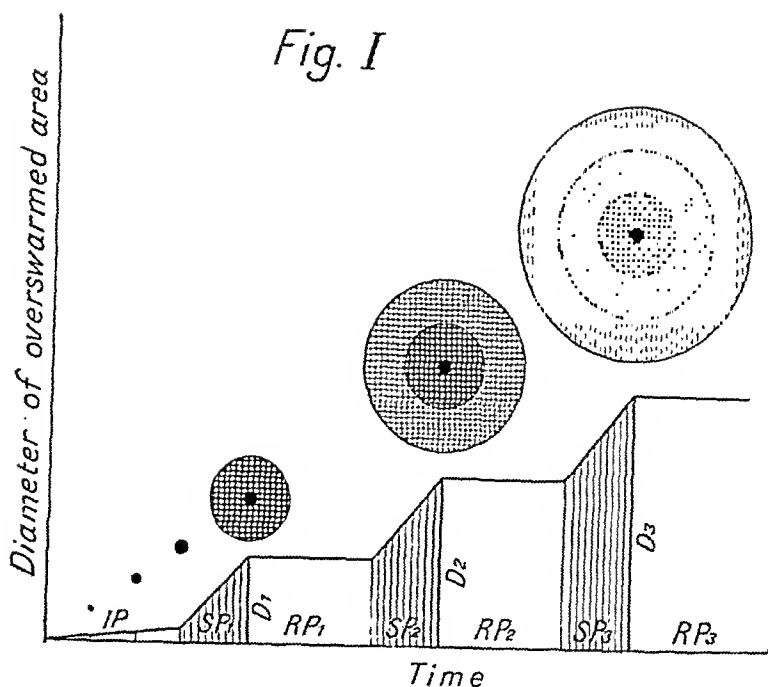
wide strips were cut out of the agar the *Proteus* swarmed on, from the centre of the colony to the edge. Impression preparations were taken from the surface on to a slide. The preparations were dried, flame-fixed, stained with carbol-fuchsin and microscopied from one end to the other. The earlier findings were verified — uniform microbes in the centre and variable morphology and staining of the individuals towards the edge. In some preparations with predominantly long forms in the periphery it looked as if the microbes lay parallel with the longer axis in radial direction. It often happened that certain individuals seemed to be so long that they stretched over the whole field of vision.

For the study of the microbes *in situ* thin slices of agar were placed upon a slide (Ørskov's technique), inoculated and put in a moist chamber at 37° C. At short intervals the preparation was observed under the microscope and it was seen that long, rod-shaped microbes suddenly began to crawl and wriggle out of the colony that had appeared. The same phenomenon could be observed on ordinary thin agar plates.

For further studies of this phenomenon, the following technique was used. A slide was placed on the bottom of each of a series of petri-dishes and these were then filled with varying quantities of melted agar. In the thinnest, the slide was only just covered, while in the thickest, a 2—3 mm. cover was placed over the slide. An inoculation was made on one end of the slide and to the side of the other end. In this way, we obtained one colony which grew and started swarming on the thin layer of agar over the slide and another colony which lived on the thicker layer of agar beside the slide and sent its swarming forms over onto the slide. The slide was then cut out and placed on the stage of the microscope.

Experiments now showed that if a plate is inoculated with *Proteus* culture, a colony grows in the course of an initial period, (I. P.). From this colony long rods suddenly begin to wriggle forward. This means that the microbe's first swarming-period has begun. During this period the colony's diameter grows to D_1 . (Fig. 1). The microbe's advance stops more gradually than it started and in this rest-period (R. P.₁), the width of the overswarmed zone remains unchanged. Then long forms again begin to wriggle forward from the edge and in the next swarming-period (S. P.₂), a new zone is formed and the diameter of the colony increases to D_2 . A new rest period (R. P.₂) follows, and after this, a new swarming-period (S. P.₃), which leads on to D_3 , etc.

The following experiment was then carried out in order to give some idea of how separate individuals behaved and looked during a swarming- and a rest-period. At 15 min. intervals, a small rectangle was cut out of a plate-culture, from the edge of the zone, during the whole swarming-period, (S. P.₂) and rest-period (R. P.₃). Impression preparations were taken, dried, flame-fixed and stained with carbol-



fuchsin. During the whole swarming-period, it was found that practically all the microbes were long and homogeneously stained. At the beginning of the rest period, the long rods became blue-black beaded. A quarter of an hour later, a number of the long forms had divided. After another half-hour, in the rest period, we found a large number of short rods which usually contained two granules; here and there longer forms with several granules and a number of moderately long forms without granules. A quarter of an hour after this preparation was taken, there were a large number of long rods, homogeneously stained, and at this point a new swarming-period began.

From these observations, it was fairly clear that the microbes had a life-cycle and that swarming and rest were closely connected with definite periods in this cycle.

That little attention has been paid to this interesting phenomenon is evident from the fact that up to the present most handbooks in bacteriology have stated simply, with regard to this microbe's morphology, that it is a gram-negative pleomorphic rod, which contains numerous flagella and is therefore very mobile and capable of swarming on a solid surface.

More than this, I myself, did not know of the matter when I started on the above-mentioned experiments. It seemed to me, however, extremely doubtful that my observations could be quite new — a doubt which proved to be justified. A number of works on this subject were found to exist and it appeared that on the whole, the different research-workers had had the same findings, but none of

them had made a closer study of the cycle of the cellular development and those observations and interpretations which had been made by certain of them were not consistent with my findings. I therefore considered it might be useful to go ahead with a more thorough investigation into the problems connected with the swarming of the proteus. In order to save space, I have decided to give an account of my own experiments first and to postpone the survey of the works of other workers to the last section of the paper. The work was purely experimental and can be divided into three sections:

- Section I: The relation between time and the different manifestations of life of *Proteus* in different surroundings.
- Section II: The cellular development under different conditions of life, and attempts to clarify the significance of the above-mentioned granules.
- Section III: A study of the swarming itself, (movements of the microbes on different media).

Section I.

The first objective was to attempt to clarify the different spreading-phenomena shown by proteus during routine work. The usual media were therefore chosen for the work and only their dryness was to be varied. In addition, the influence of the temperature on the microbe's life-cycle was to be studied.

Media:

A detailed account of the media will be given under the separate experiments. It may be mentioned that, as in the other experiments described here, all the plates for parallel studies were made equally thick. The degree of dryness was determined either in % agar, or by different drying and storing of otherwise similar plates.

Temperature:

The observations were made at 37° C., 30° C., approximately 20° C. and at room-temperature (15°—18° C.)

Proteus Strains:

Experiments with five typical proteus strains showed that they all behaved rather alike as far as swarming was concerned. One of these strains, O. M. S. 43/46, is used in all the experiments described below.

Technique:

Unless otherwise stated, the inoculum was taken from an 18-hour old plate-culture and incubated at 37° C. A small drop of a suspension was placed with the same loop in the centre of each plate. Some of the plates were small (100 mm. diameter) and others large (200 mm. diameter). In some experiments, special vessels were used for the cultivation. At regular intervals, the cultures were examined and the diameter of the colony measured in mm. The result of the observations is shown as a function of time and growth, expressed by the colony's spreading (diameter), measured in mm.

Some experiments are shown in the following diagram.

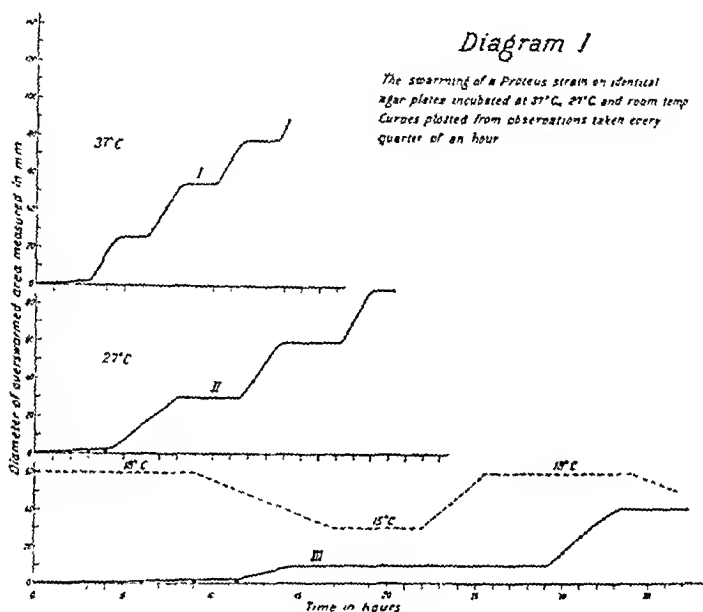


Diagram I.

Medium/ Brom — thymolblue — lactose agar 2½ % in 100 mm. plates which after drying 18 hours at 37° C. were inoculated in the centre.

I. Plate inoculated at 37° C.

II. » » » 27° C.

III. » » » room-temperature, which, during the day, was kept at approximately 18° C., but at night dropped to 15° C.

This experiment shows that the I-period increases in length with a falling temperature. At 37° C. it was 3 hours, at 27° C., 4 hours and at 18° C. it had increased to 11½ hours. If the three curves are compared, it is further found that there is relatively little difference in the S-periods and that the zones are almost equal in width, apart from the first zone at 18° C. The reason for the dissimilar general ap-

pearance of the curves is the R-periods, which, like the I-periods, increase greatly in length with a falling temperature.

The curves show that changes in temperature within a certain limit (37° C. — approximately 18° C.) have comparatively little influence on the swarming-periods, either as regards length of time or width of zone, but a strong influence on that part of the life-cycle which takes place in the R-periods. The significance of this on the study of the cells will be referred to later.

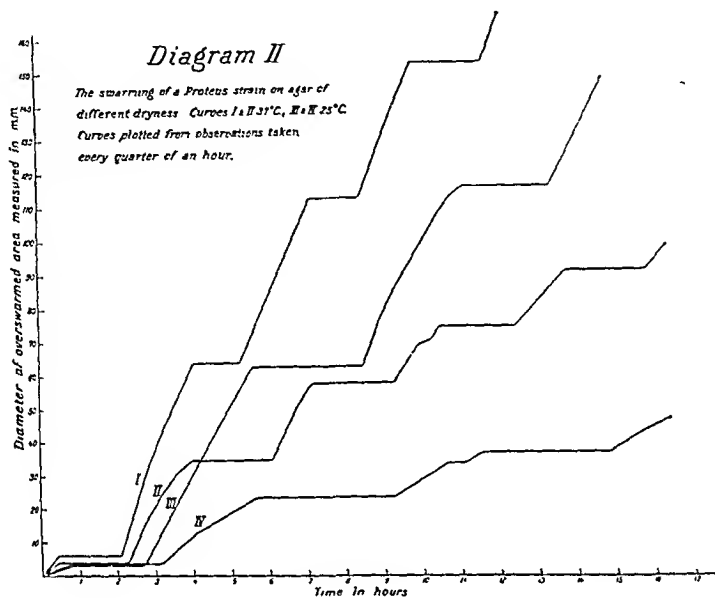


Diagram II.

For this experiment, media of different dryness were used and incubation was at 37° and 25° C.

Media: $2\frac{1}{2}$ % agar in 4 200 mm. plates. Plates I and III were made the day before the experiment and dried for 20 hours at 37° C. Plates II and IV were prepared 8 days before the experiment, dried for 40 hours at 37° C. and stored approximately $6\frac{1}{2}$ days at 4° C.

All the plates were inoculated in the centre with a suspension from a 10-hour, 37° C. agar plate-culture. Plates I and II were incubated at 37° C., plates III and IV at 25° C. Some of the inoculum was immediately found to swarm and form a small zone, so that there was no pure I-period in this experiment.

The influence of the temperature is evident from curves I and III, which behave as expected from Diagram I. The same applies to the relationship between curves II and IV. Of interest in this experiment is the relationship between curves I and II, and II and IV — i. e. the significance of dissimilar degrees of dryness under otherwise similar conditions. The result seems fairly obvious — with in-

creased dryness of the medium, the R-period is prolonged and the width of the zone decreases.

Further experiments, with variations in the degree of dryness of the media, showed that in this way it was possible to obtain varying pictures of growth on the plates — from the damp, fresh plates which after a few hours at 37° C. were covered with an even, thin film of the swarming microbes, to the plates which with increasing dryness of the agar showed slower and slower growth and increasingly narrow zones, which were found as clear steps down towards the periphery. If a relatively dry plate with slow growth was not protected against further drying during the experiment, the breadth of the zone could be seen to decrease and the growth (spreading) to stop far from the edge of the plate.

As was mentioned earlier, one *Proteus* Strain only was used for all the experiments and was therefore cultivated for quite a few generations. It was noticed that in time this strain became less exacting and grew more easily in unfavourable conditions than it did when fresh. After a year's cultivation, it formed an even, continuous film on the media on which fresh strains form clear zones.

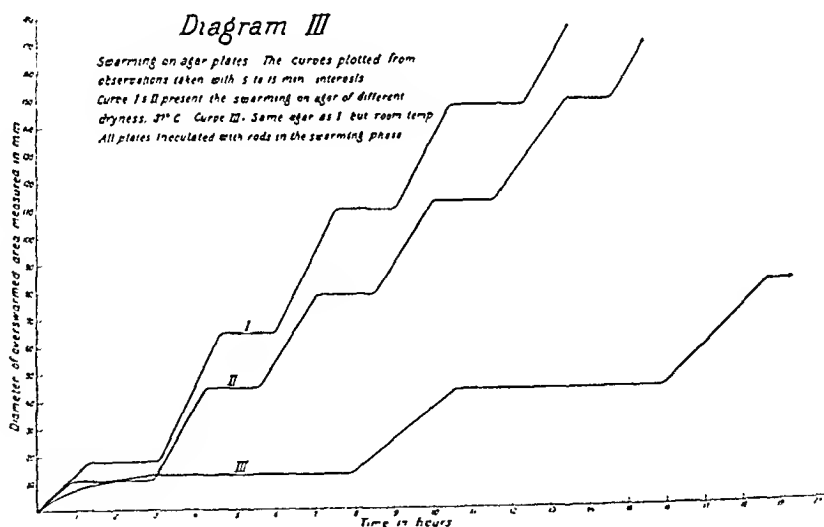


Diagram III.

Medium: Brom-thymolblue lactose agar 2½ %.

200 mm. plates.

Plate I and III dried 20 hours at 37° C.

Plate II dried 20 hours at 37° C. and stored 6 days at 4° C.

Inoculum: Microbes in the swarming stage.

A number of experiments, including the one described in Diagram II, showed that the inoculum might contain cells which were capable of swarming immediately. In this experiment the inoculum was taken from a section which at that moment was full of swarming microbes. It was *Proteus* plate-culture at room-temperature which, sometime before the inoculum was taken, had arrived at R. P. 3. With a microscope it was possible to ascertain that at the edge of the zone all the cells were at rest, but 7—8 mm. towards the centre it was full of long, mobile rods. It was from this section that the inoculum was taken.

As was expected, the inoculum swarmed immediately. Practically the whole culture crawled outwards, rather quickly at 37° C. and somewhat more slowly at a lower temperature.

The difference between curves I and III bears, as one might expect from Diagram I, the »stamp« of temperature. On comparing curves I and II one finds that in the first phases, the difference lies mainly in that I has a larger breadth of zone than II, a variation which, as Diagram II shows, is typical of an unequal degree of dryness of the medium. In the later phases of the experiment it was found that in the case of Plate II the R-period became shorter and the zone-breadth greater. The cause of this proved to be the shape of the bottom of the plate. This had a hump, highest in the centre, so that the agar increased progressively in thickness from the centre to the edge of the plate and as this layer of agar increased in thickness, so the drying naturally decreased. Thus, each time the microbes advanced, they found continually better living conditions. It is this that one must suppose is expressed by the changes in the curves.

The experiments described showed that as long as the nature of the culture media was uniform, the zone-breadth seemed to be constant. The question then arose as to whether it was the number of microbes capable of swarming which was the decisive factor in determining the zone-breadth, whether it was the conditions of the surface of the agar which were most important, or whether it was a combination of both these factors.

On microscopical examination of a colony swarming on agar, it looked as if the microbes crowded together with the same density per square unit from zone to zone. If this were the case, one should be able to assume that the number of swarming microbes from one zone to another was proportioned to the area in the corresponding zones. In these experiments it would, for example, be conceivable that the number of microbes capable of swarming increased from zone to zone, but that this was not expressed in an increased zone-breadth because of the spread in radial direction, so that the area of the zones increased proportionally to R^2 . In order to solve this problem, the following experiment was carried out:

A 5 mm. thick layer of 2 % agar was poured into a glass box 30 cm. long, 4 cm. wide and 1.2 cm. high. The »plate« dried for 18 hours at

37° C. The inoculum from an 18 hour, 37° C. plate culture was placed in a stripe right over the agar, approximately 2 cm. from one end. The »plate« was covered with a glass lid and all the edges covered (sealed) with plastiline and placed for incubation at 30° C. Evaporation and drying were thus prevented and the microbe forced to swarm along a track with a constant area per unit of length. The question was whether, under these conditions, the zone-breadth would remain constant. The experiment was carried out in a room at 30° C., and the plate lay the whole time under a microscope. The bottom of the plate faced upwards and on this was stuck a strip of mm. paper, so that the advance of the microbes at a given time could be read accurately, and definite zones in the overswarmed region could be examined at regular intervals.

The first zones were regular, both as to time and zone-breadth. Gradually, however, the S-period increased in length and the zone-breadth became larger, and at the end of the track, the microbe swarmed continuously without any R-period. Observations carried out during this experiment explain this condition. It had previously been assumed that the long rods that were capable of swarming were only developed once on a definite section of the agar — a supposition which is perhaps fairly correct so long as one is dealing with a dry medium. The agar used in this experiment was fresh and comparatively soft, and it was possible to ascertain, on microscopical examination, that the »swarmers« were developed in several relays in the same section and that they formed secondary (tertiary, etc.) waves and zones across the agar. This was, of course, found to create disorder in the periodicity expressed by the advance of the edge. 9 hours after the inoculation, the swarming had reached a point 73 mm. from the »starting line« and it was possible to observe swarming microbes all over this region. 14 hours after the inoculation, the swarming had reached 117 mm. from the inoculation. In the first 50 mm. of the track there were, at this juncture, from no swarmers to only a few; further out, variable activity of swarming microbes right to the advancing edge. 24 hours after the inoculation, few or no swarmers were to be found on the first 125 mm. of the course, and from there out towards the advancing edge there was changing intensity of swarming, but nowhere complete rest.

It is due to these secondary (possibly tertiary, etc.) waves of swarmers that on soft, fresh agar, proteus forms an even film. On a dry plate »steps« are found, because each zone is able to breed one generation only of swarmers and afterwards only stationary forms.

The first zones in this experiment showed that the breadth remained constant even if the square of the overswarmed region did not progressively increase in the same way as with spreading from the centre of a round plate. The density of the microbes per area does not, therefore, seem to be a decisive factor as regards the zone-breadth. In all probability, this depends upon the rate of movement of the

microbes — (which, in its turn, is dependent on the nature of the medium) — and upon that time in the cell's cycle of development which is connected with the S-period.

Section II.

As already stated, the result of the preliminary experiments suggested that *Proteus* had a life-cycle which it would be of interest to study more closely, and those granules which appeared regularly and quite suddenly in the long »swarmers« naturally stood out as representing one of the central problems.

That the choice of stain should have fallen upon carbol-fuchsin, was fortunate. With this method the granules became very clear; with a stain such as, for example, methylene blue or Gram's method, the granulation is not seen so distinctly. In the study of the microbe's morphology during the different phases of its development and life-cycle, we used chiefly (apart from living microbes) impression preparations, which after air-drying and flame-fixation were stained for 45 seconds with diluted carbol-fuchsin.

The microbe's cycle of development falls naturally into three stages:

Initial period (I. P.)

Swarming period (S. P.)

Rest Period (R. P.)

each of which will be discussed separately.

Initial Period:

The *proteus*-strain O. M. S. 43/46 was inoculated in the centre of a 2 % agar-plate, which was incubated for 18 hours at 37° C. This spread out evenly over the whole plate, which was then kept for six days at room-temperature. A number of strips (c. 10 × 3 mm.) were cut out of this agar-culture and these were used to inoculate a fresh agar-plate with careful impression. Each strip was used for one inoculum only, and each inoculum was placed on the same plate. In this way the inoculum became fairly uniform and the conditions of growth the same for each inoculum. One of the inocula was immediately cut out and the preparation was made by the »impression method« — this was preparation no. 1 and shows the microbes in the condition they were when inoculated. The culture was then incubated at 37° C. After 1 hour 45 mins., preparation no. 2 was taken and after that, other preparations followed at intervals of about 15—20 minutes until swarming had started.

The majority of the cells in the inoculum were thin, short and badly stained. Scattered among these pale microbes, strongly stained

bodies were found, varying from spheric to short rod-forms. In those preparations which were taken at definite intervals after the culture had been left for incubation at 37° C., the cellular development could be followed step by step. A film of the same weakly stained cells, as were found in the inoculum and which one must presume to be dead, — (ghost cells) — also form a base in the first preparations taken after incubation began, but even after an hour, thick, partly fusiform strongly stained rods of somewhat variable length are to be found scattered amongst the ghost cells. A number of these vigorous cells have strongly stained intracellular granules. Some individuals appear to contain only one granule, which in some cells seems to be in division. Slightly longer rods have more granules. The further development in the first place consists chiefly in the reproduction of the type of cell that has been described. In thin parts of the preparations, the new cells are found in small groups, and from one preparation to another it is possible to see that the group has grown. After some hours' incubation, one finds thin, homogeneously stained rods of variable length appearing in among the thick, stout, granulated rods. After a short time, a large number of these homogeneously stained rods are found to have grown to a great length and they completely dominate the picture. Ghost cells can only be seen here and there in the preparation, and the same applies to the shorter, granulated rods. At this point, several »control« colonies begin to swarm.

Swarming Period:

So long as the conditions of growth are unchanged (medium, temperature), the preparations which are taken from the edge of the colony during a swarming period show a uniform picture.

In a fresh culture at 37° C., predominantly long rods are to be found. A large number of these are homogeneously stained, a number have irregular granules or cracks seem to appear in the microbe here and there. In some long, and in the short forms which are found occasionally, the granules arrange themselves in regular transversal bands. The proportion between the homogeneously stained and the granulated rods may vary considerably from one preparation to another if the technique is not accurate and uniform. The reason for this is, that in the peripheral part of a culture in S. P., one finds both cells which have settled and cells which swarm towards the free agar surface over and between those at rest. If one cuts out a piece of agar from the edge and takes a very light impression, one finds predominantly long homogeneously stained rods. If the preparation is taken with insufficient care, however, one gets also a larger or smaller number of granulated rods of variable length. If one makes, for example, three impressions of the same piece of agar beside each other on a slide, a definite variation in the cells is found from one impression to

another. In the first, one finds overwhelmingly homogeneously stained rods, for it is the swarming cells which were the first to lose hold. In the second, and still more markedly in the third, impression, the rods are of variable length and practically all contain more or less organized granules. Here one finds predominantly the cells which had settled. In brief, the swarming cells seem to be fairly uniform in size, homogeneously stained and without intra-cellular granules. A new phase in the development of the cells commences from the point when they have settled — or they settle quickly at 37°C . and more slowly at a lower temperature. From a culture at e.g. 22°C ., practically only long, homogeneously stained rods are to be found during the whole S. P. in the peripheral part.

Rest Period:

Diagram I showed that the whole R. P. increases with falling temperature. From this it appeared that the different stages in the cellular development must occur more slowly, and more circumstance could be utilized in the experiments. The following method was used:

A 2% fresh agar-plate was inoculated in the centre and left for incubation at 27°C . After some time in S. P. 3, the central part of the colony was cut out so that an approximately 3—4 mm. broad strip which had formed during the last 15 minutes, were found the cells could swarm into this area. After about 15 mins. the strip had grown to a width of 8—10 mm. and with the microscope it was possible to see that practically all the cells had settled. The most central cells should therefore be about half an hour earlier in development than the most peripheral. From this strip pieces of agar were cut at regular intervals for impression preparations until the beginning of a new S. P.

After this experiment had been carried out several times, it was clear that the development was constant, apart from some variation in time. The development must therefore be described without reference to definite lengths of time.

A short time after the long, homogeneously stained «swarmers» have settled, the irregularly arranged intra-cellular granules appear. It looks as if the microbe has made cracks here and there. After some time, the granules give a beaded appearance. The next step is a contraction in the middle of one or more granules and the microbe divides itself between these.

At one stage, long, medium and short rods are to be found mixed.

A little later on, few or no long forms with regularly arranged granules are to be found, but all are short and contain two or a few granules. The short rods have one granule at each end and each of these therefore represents half the transversal band.

At a point when the preparations show practically only short, granulated forms, some long forms are still to be found, but these are without arranged granules and do not stain well. They are probably pathological or dead cells — a matter which will be dealt with in more detail on another occasion.

As the development continues, short, homogeneous forms will soon appear among the granulated ones. These are new swimmers beginning to grow once more.

The development in question occurs during the colony's R. P. — a designation which is therefore not applicable to the cells; for these, it must be regarded as a period of multiplication and differentiation.

Parallel with the study of the life-cycle of *Proteus* growing on agar surface there was also carried out a study of the development of the cells in fluid medium, namely broth. The broth culture was incubated at 37° C., and with one hour intervals the growing cells were studied both in stained preparations and in living preparations.

The cellular development seems to be exactly the same in fluid medium as on agar surface. In preparations taken after 6 hours incubation there were found lots of swimmers in a mixture of cells at all different stages of division. But naturally in such a mixture it is very difficult to follow the development step by step as on the surface of a solid medium.

Mechanism of Division and the Intra-cellular Granules:

As is evident both from stained preparations and, still better, from direct microscopic study of the swarming microbes on the agar-plate, all the individuals in this phase are of great length. There are certainly some variations, but no short forms swarm. The length may in the least exceed 30 μ m. The number of organized granules varies with the length. There may be 16 — and perhaps still more. It cannot definitely be stated that an even number of granules is invariable, but it looks as if this should be the case with microbes with quite regularly arranged granules. Occasionally one finds cells with irregular spaces. It may be that the distance between the granules is unusually great — or that rather large numbers of granules have collected on one section. It is difficult to know whether this is because the arrangement was not finished when the preparation was taken, or because there are cells with some abnormal or pathological links.

The division of the microbe takes place in the middle of the granules. The single individuals which originate from a long swimmer in this way have a granule at each end, and each of these represents

half a single granule in the original cell. A somewhat remarkable phenomenon occurs at the ends of the swarmer; here too, division takes place in the middle of the terminal granule — so that the peripheral stump which breaks off thus gets only half a »dose« of granule in comparison to the other links. In certain cases it looks as if this »end piece« consists of one granule only — in other cases it has acquired some protoplasm. It is not improbable that the further division of the segments with a granule at each end occurs in such a manner that such »monogranular« individuals again break off at the ends.

What happens to the end-pieces — whether they are not capable of living or whether they are very much capable of living — is not known. Nor is it impossible that it is from these that the long swarmer originates.

The granules might be thought to represent:

1. Glycogen.
 2. Compressed cytoplasm on account of fat-deposits.
 3. Volutin.
 4. Nuclear apparatus.
1. Since it was not possible to demonstrate the granules by staining with Lugol's solution, glycogen could be excluded.
 2. The extreme precision and speed in the formation of the granules would hardly seem to be the result of pressure from fat bodies — which are usually variable both in shape and size (I. M. Lewis). Fat bodies cannot be stained with water soluble aniline stains. If it had been fat, these granules, which in this case would have represented compressed cytoplasm, would have been equally clearly visible with any aniline stain. This was not the case. When stained with e. g. methylenblue, the microbe appears almost, if not quite, homogeneously stained at all stages.
 3. The systematic arrangement speaks also against volutin. As far as the shape and arrangement of the volutin granule go, the same applies as to fat. The decisive test was that the granule was insoluble in water at 80°—100° C., so that volutin could be excluded.

The glycogen-, fat- and volutin-contents in microbes have been studied thoroughly by many bacteriologists. In his survey and table of the microbes which form the three cell inclusions mentioned, I. M. Lewis has not included *Protens*. Franz Neuman, who has made a close study of cell inclusions in *Protens*, maintains that this microbe never forms volutin.

4. Nucleus:

The impressive system and order which was found in the formation and division of these granules naturally led on to the question of whether it was a bacterial nucleus that they represented.

By using Giemsa stain and carbol-fuchsin on preparations which were taken simultaneously, it was found that at the stage when

the carbol-fuchsin preparation was homogeneously stained, the Giemsa preparation was also homogeneously basophilic. A preparation taken at a phase when the rods, stained with carbol-fuchsin, were seen to contain regularly arranged granules, showed that rods stained after Giemsa had alternately eosinophil and basophil segments — a condition analogous to nucleated cells.

Feulgen-Rossenbeck's reaction for desoxyribonucleic acid is now thought to be probably a decisive criterion in the question of whether a nucleus is present or not. A number of bacteriologists and botanists who have worked with this reaction, have shown that *Proteus* belongs to the microbes in which intra-cellular bodies containing desoxyribonucleic acid may be observed.

By the use of this reaction, it was found that the swarming forms homogeneously very weakly blue-grey stained, so clearly, that the microbe was easily visible under the microscope though not sufficiently clearly for photographic reproduction.

In other phases of the development, regularly arranged granules were found quite analogous to carbol-fuchsin and Giemsa preparations — (photo). It is found that only the granules are stained and the rest of the microbe is only just perceptible as a boundary against the surroundings. The conclusion from this must be an assumption that in the swarming phase the microbe has its desoxyribonucleic acid, its nuclear material, distributed in the protoplasm, that this collects and arranges itself in segments in which division takes place.

One might give a tentative explanation of the condition of the nucleus of the swarmer by assuming that the latter originate from the above-mentioned »end pieces«. Their gene material would then consist of only half a dose of nucleus and possibly be incapable of division. Instead they grow into the long swarming rods. During swarming an exchange of nuclear material conceivably might occur between different »monogranular« cells, which after this process become capable of division.

The cellular development may also be studied to some extent in living microbes. Instead of a slide placed in the bottom of Petri dishes, we used large cover-glasses which were covered with a very thin layer of agar. After 3—4 hours drying at 37° C., the agar was inoculated beside the cover-glass. When the swarming zones had reached a point some way over the cover glass, this was cut out and the overswarmed region covered with a thin cover glass. Such preparations were sufficiently thin to be studied in dark ground illumination.

On the whole, the swarming forms looked homogeneous, but in some intra-cellular, stronger light-refracting bodies were to be seen. By this method it was also found that the microbes in R. P.

did not remain quite still. Now and then they moved backwards and forwards a little, changed position or turned slightly. In good preparations, it was possible to see intra-cellular vibrating movements and peristaltic contractions along the microbe. The intra-cellular bodies eventually become as orderly as gleaming pearls on a string, and division often occurs in several places. Some cells which are practically divided, may move round somewhat within the field. Then it often looks as if the separate fragments are not co-operating properly and the thin »bridge« which holds them together thus gets severed.

Section III — Swarming.

(Movements of the Microbes on Agar Surface.)

Method: Either a slide or a large cover glass embedded in agar-plates. Inoculated as described above. Experiment carried out with cultures incubated at different temperatures (e. 22° C., 27° C. and 37° C.). During a suitable phase of the spreading, the slides were cut out, placed on the stage of the microscope. The swarming was observed with a dry lens — 80—600 times magnification — and oil immersion and dark ground at magnification of 900—1500 times. The phenomenon is very interesting and exciting. It is difficult to describe it and the only method of demonstrating this teeming life, for those who personally have no opportunity to spend time or energy on their own experiments, is to use films. As shown in Section I, diagrams II and III, the state of dryness of the medium plays the biggest role in determining the width of the swarming zone, which becomes large on soft plates and decreases with increasing dryness.

When a colony swarming on a fresh, comparatively soft agar is placed under a microscope with magnification of about 80 times it looks as if numerous tongues protrude from the edge. They stretch outwards and often tear themselves quite free. They sail a little outwards like an ice-floe, almost always to direct their course back again towards the mother colony. In their wanderings, they leave behind them small dots here and there, whilst the main mass spreads out and settles just under the edge. Some »tongues« may go right back and re-establish contact with the mother colony. Certain writers have most aptly compared this picture with coastal scenery, with fjords and numerous islands outside.

When a tongue is observed with magnification of 4—600 times, it is strange to see how all the individuals lie in one direction. Bent around the tip of the flock there is usually a long rod — as if intended to prevent splitting. During swarming, individuals fall away, in some

places in small groups, in others rod after rod arranging themselves in line, so that it looks like a long filament. (Such chains in the impression preparations may lead to confusion and be mistaken for thread-shaped forms). It often happens that most of the individuals are left behind, whilst a few rods, or perhaps only one, drive further with a cross-lying rod in front of the »bow«. If two or more groups collide, it may happen that the one steers right through the other without changing course. A number of individuals may part from the one group and follow the other. A common phenomenon when two groups bump together is that they drive on in a circle for a time. While a large group is swarming in a definite direction, one or more rods may suddenly be seen — usually in the middle of the group — beginning to drive back right against the stream.

In an incredibly short time, both larger and smaller groups have spread over, and covered a field of vision.

On a dry agar, it looks as if the microbe has greater difficulty in spreading. Few or no tongues stick forward from the edge, according to the degree of dryness. It is possible to see that the swimmers wander backwards and forwards in a circular course by the edge and seem to roll and push out sideways. Seen with low magnification, the edge of the colony may become somewhat wavy, but one sees no »islands« outside, as one does on soft agar. The strange phenomenon that detached groups always wander back towards the mother colony should signify that the colony prepares areas around itself somehow or other and that the swarming cells turn round when they emerge from this region. On very soft agar, it is possible to see that some small groups quite lose contact with the mother colony and sail several cm. forwards over the plate before settling.

The single microbe's movement and the question of which organs it uses to crawl forwards are of the greatest interest. In this connection the discussion must lead on to the subject which, at the moment, is very topical — the significance or very existence of the flagella. A number of observations in this field will be treated in another work.

Historical Survey.

In 1885, G. Hauser described a microbe he had isolated from putrefying meat. On account of the very variable morphology shown by this microbe, Hauser called it *Proteus*. He described three different types: *Proteus Vulgaris*, *Proteus Mirabilis* and *Proteus Zenkeri*; but after having studied the microbe more closely he came to the conclusion that the two last were actually only variations of the first.

Hauser cultivated the microbe on gelatine plates and found that it possessed the following properties:

1. It liquified gelatine.
2. Its morphological appearance varied from short rods to long, thread-formed rods.

3. The long thread-forms were capable of spreading over the medium. Swarming, as he calls this phenomenon, he states to be one of the characteristic properties a microbe must have in order to be classified as *Proteus*.
4. The long rod-forms he found at the edge of the colony; in the centre, he found only short forms.

Hauser has given a classical description of the swarming cells. The fact that he was unable to follow more closely either the cellular life-cycle or, in connection with it, the periodicity in swarming, is certainly due to his not knowing of agar as a medium. When cultivated on gelatine, which *Proteus* liquefies, the periodicity in swarming will be but little marked. It is, however, probable that Hauser was also aware of this phenomenon. He has also described intra-cellular bodies in the long rod-forms and bipolar granules in the short.

Despite the fascination of this phenomenon of swarming, there are very few bacteriologists who have given the subject any closer study.

O. Moltke, who in his monography on *Proteus* (1928) gives a detailed account of the microbe's biochemical and serological contents, treats the morphology of the microbe far less thoroughly. O. Moltke verifies the pleomorphism Hauser described as so characteristic of *Proteus*, and by cultivating the microbe on agar, showed that only the long rod-forms swarmed. Moltke came to the conclusion that the swarmers occur in a colony when the condition of life are no longer optimal — (accumulation of toxic products — deteriorating, worse conditions of nutrition). The swarming itself Moltke has treated in great detail; the cellular development, on the other hand, he has not paid particular attention to.

Russ-Münzer, who says that Hauser has dealt with swarming so accurately that nothing but confusion has resulted from all further treatises on the subject, raised the problem, as to why the swarming *Proteus* forms zones. To solve it, she used the following technique: The centre of an agar-plate was inoculated with a 24-hour *Proteus* culture. The zone's outer limit was marked on the plate every hour. In this way, she found out the periodicity in swarming and states that at 37° C., it takes 4 hours from the beginning of one swarming-period to the beginning of the next, with approximately the same length of time for swarming and rest. At a lower temperature — e. g. at room temperature — she found that it took about 24 hours from the start of one swarming period to the next. She does not state whether the prolongation falls in the swarming- or the rest-period.

In the long rods, Russ-Münzer found regular more strongly stained parts, and states that the microbe seems to be constricted at the strongly stained parts. She touches on the question of whether the granules might represent the nucleus, without, however, discussing it in more detail. The homogeneously stained phase is not mentioned.

Russ-Münzner believes that the swarmers are a product of changes in the milieu and that their »purpose« is to direct the microbes over to new »pastures«. When swarming has reached the edge of the plate, one no longer finds the long rod-forms, »for then they no longer have any purpose«. She thinks that long rod-forms are not developed in a fluid medium, for here too they have no »purpose«. This last assertion must rest upon theoretical considerations rather than on experimental studies. Further, this writer believes that the type of cell-forms found in the inoculum has no influence on the swarming-period. This is consistent with the findings in experiments described in the first section of this present work, if one does not take the initial period into account.

The granules which are found in *Proteus* have engrossed many bacteriologists. Some, or perhaps most, of them, if one goes back some years, have interpreted granules as involution-forms, whilst during recent years, the interpretation has centred increasingly around the nucleus.

Franz Neuman draws the following conclusions from his experiments on the nucleus contents of bacteria: Firstly that in young cultures of *Proteus*, *pyocyaneus*, *coli*, *bact. mycoides*, *anthracis* and *Azotobacter chroococcum*, chromatin can be detected in the form of a nucleus-like structure, and secondly that no cells capable of dividing are to be found without chromatin.

Largely on account of his studies of *Proteus* with Feulgen nuclear-reaction and in Giemsa preparations, Neuman came to conclusion that he could »state with certainty that division of the nucleus-substance in diffuse morphologically indetectable form never occurred.«

That Neuman was not aware of the *Proteus* cell in the homogeneously stained phase must be due to the fact that he did not know of the cell's life-cycle and that he always took his preparations a definite time after the making of the culture, at a stage when he would always find granulated forms. As he believes that the cells that are capable of dividing must have chromatin arranged in the form of a nucleus, it is possible that he has been aware of the homogeneously stained cells and interpreted them as being incapable of dividing.

Discussion.

The microbe dealt with in this work, *Proteus* Hauser, is named after its ever changing morphology and colony appearance, and this property has also been the subject for the present study.

Variation in the morphology of a microbe, owing to changes in conditions of life, is a natural phenomenon. It must also be expected that factors which exert an influence on the single cell must also influence the entire population — the colony. Therefore, a description of the morphology of a microbe and its colony appearance must necessarily be accompanied by particulars about the conditions of life.

This study has indicated that *Proteus* under appropriate conditions has a life cycle and that variations in the appearance of the cells correspond to certain steps in the development. Under unfavourable conditions as in a broth culture which has been incubated for several days, the living cells appear as very short rods only, or more or less as coccoid forms. In preparations from such a culture the well stained living cells are found scattered among pale ghost cells. From such conditions brought onto a favourable medium, the cells to start with repair and prepare their general condition, and then commence a simple division. If the conditions of life are sufficiently good, the cells produce a new type of offspring — the swarmers.

Some confusion seems to be connected with the swarmers. Thus, O. Moltke came to the conclusion that the swarmers occur in a colony when the conditions of life are no longer optimal. Also Russ-Münzer believes that the swarmers are results of a deterioration in the milieu and that their »purpose« is to direct the colony to new »pastures«. Russ-Münzer even states that the swarmers are not developed in fluid medium, for there they have no »purpose«. This last statement is definitely not correct. (See Section II).

In this study one came to another conclusion than those of Moltke and Russ-Münzer regarding the swarmers. As shown in Section I, the swarmers appear and in larger numbers on a favourable medium, and even in several relays in the same area. In a less favourable medium, one must presume that the conditions of life deteriorate more quickly than in a better one, and according to the opinions of Moltke and Russ-Münzer, it should be expected that the swarmers would develop more quickly and in larger numbers in such a medium, but comparisons proved this not to be the case. Also the fact that toxic substances (phenol, narcotica) in small doses prevent the development of the swarmers, whereas much greater doses are required to prevent growth altogether, shows the swarmers to be the most delicate cells.

One of the central problems in this study has been the granules which suddenly appear in the swarmers, and also are found in the short cell forms during the growth period. Already Hauser observed intracellular granules, and explained it as an involution phenomenon. Up to the latter years, most bacteriologists who have dealt with *Proteus* give the same explanation.

By the Feulgen-Rossenbeck nucleal reaction, electron-microscopic studies and other modern methods, strong evidence has been produced that the bacterial cells are nucleated. (Piekarski, Caspersson & Co., Knaysi & Co., Tulasne & Vendrely). With these methods, a number of authors have examined *Proteus* and found this microbe nucleated.

In the present study also, the conduct of the nucleus, (the granules), at the different stages of the described life cycle has been dealt with. It has been shown that the swarmers in the actual swarming phase are homogeneously stained (Feulgen stained), which means that

the desoxyribonucleic acid must be present in diffuse, morphologically undetectable form. Before division, the swarmers become beaded, and the division takes place through the middle of the granules. At the ends of the swarmers, a somewhat remarkable phenomenon occurs. Here too the division takes place in the middle of the terminal granules, so that the peripheral stumps which break off receive only half a dose of granula in comparison with the other links. What happens to the monogranular end-pieces has been discussed.

The movement of the microbes on the surface of agar has been dealt with briefly. This field is being subjected to further study.

Summary.

1. Studies on pleomorphism and swarming in *Proteus* are reported. Evidence is presented that the microbe has a life cycle, and that variations in the morphology in the individual cells and of the colony correspond to definite phases in this life cycle.
2. The cells in old broth cultures are short and plump. After inoculation on an agar plate, a colony is formed in the course of an initial period, which the cells use to improve their general condition, increase their volume and produce intracellular granules, after which, division commences. When multiplication is well under way, new types of cells are produced — the so-called swarmers. These are long slender homogeneously stained rods, which spread out and come to rest again in the zone around the colony. On soft media the number of swarmers is large and the zones wide.
3. Swarmers which have come to rest develop regularly spaced intracellular granules. Division of the swarmers takes place through the middle of these granules, a number of short rods being formed. All short forms — except the end-pieces — have one granule at either end, which represents half of a granule from the mother cell (the swarmer). The end-pieces contain half a granule only. The short forms divide in the usual manner, and eventually produce new swarmers.
4. The intracellular granules give a positive Feulgen nucleal reaction and must be assumed to contain desoxyribonucleic acid. It must be assumed that they represent the nucleus of the microbe.
5. It is suggested that the swarmers may be related to the above-mentioned end-pieces.

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Explanation of Microphotographs.

The Method used in the study of the life-cycle in *Proteus* is described in Section II. Stripes were cut out of an agar-plate culture which had been incubated 18 hrs. at 37° C., and kept for 6 days at room temperature, and were used to inoculate fresh agar-plates with careful impression.



Fig. 1.

Preparation made from one of the inocula before incubation. Scattered among pale ghost cells were found strongly stained short rods and coccoid bodies. Magn. 600 X.

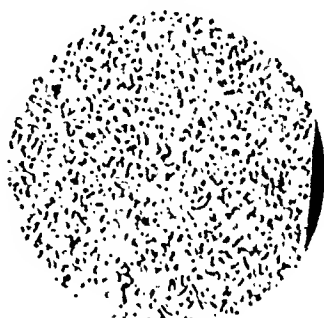


Fig. 2.

Preparation made after short time of incubation at 37° C. The well stained cells have increased in volume. Magn. 600 X.



Fig. 3.

After one hour's incubation. — Strongly stained rods of variable length are found amongst ghost cells. Magn. 600 X.



Fig. 4.

Same preparation as No. 3. Magn. 1500 X.



Fig. 5.

After about two hour's incubation the rods had produced intracellular granules. Magn. 1500 X.



Fig. 6.

In thin parts of preparation no. 5, the new cells were found in groups. Magn. 600 X.



Fig. 7.

After further incubation the groups have grown, and a new type of cell — a slender homogeneously stained rod — appears among the granulated stout rods. It is the first hatch of swimmers. Magn. 600 X.



Fig. 8.

Preparation no. 7. Magn. 1500 X.



Fig. 9.

The swarmer are increasing in size and number. Magn. 600 X.



Fig. 10.

Preparation made half an hour after no. 7. Mature swarmer completely dominate picture. Magn. 600 X.



Fig. 11.

The same preparation as no. 10. Magn. 2500 X.



Fig. 12.

The preparation taken at the start of the rest period. The swarmer have become beaded. Magn. 600 X.

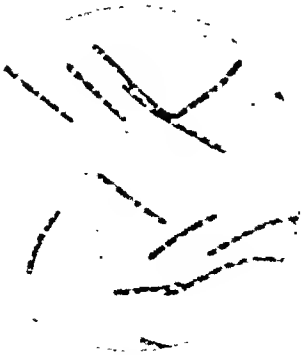


Fig. 13.

The same preparation as no. 12. Magn. 1000 X.

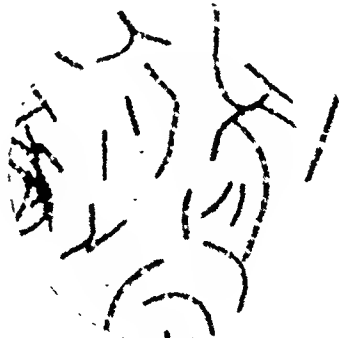


Fig. 14.

Division of the swarmer just started. Magn. 1000 X.



Fig. 15.

Preparation taken half an hour after no. 12. Division of the swarmers nearly completed. Magn. 1500 X.



Fig. 16.

A swarmer of great length containing 16 regularly spaced granules. Magn. 4000 X.



Fig. 17.

A swarmer in division at several places; also through the terminal granulum. The end-piece has very little protoplasm. Note also the abnorm length of the second link from the left. Magn. 2500 \times .



Fig. 18.

A swarmer in division. Magn. 1000 \times .

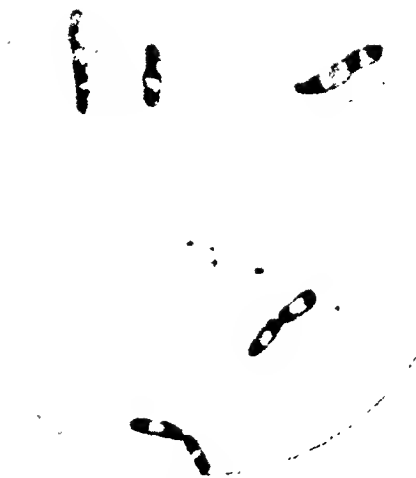


Fig. 19.

The final stage in the division of swarmer, Magn. 4000 \times .



Fig. 20.

Feulgen stained preparation taken at the same stage as no. 14. All individuals seem to contain an even number of granules.

SEROLOGICAL STUDIES ON GONOCOCCI. I. TECHNIQUE. GONO-REACTION OF »NORMAL« RABBITS. SEROLOGICAL RELATION BETWEEN GONOCOCCI AND PASTEURELLAE.

By Alice Reyn.

(Received for publication July 26th, 1948.)

Introduction.

Most workers agree that the gonococci constitute no serological unity, i. e. they can be divided into more or less constant types. Some investigators point out the problems which occur because of variability of the antigens and occurrence of transitional forms (Hermannies (1921), Torrey & Buckell (1922), Atkin (1925), Casper (1938)).

As a rule, complement fixation and agglutination tests, together with absorption, have been employed for classification of the strains.

Chemical methods have also been employed with preparation of fairly well-defined substances from gonococci (polysaccharides, nucleo-protein, glucolipoids) — e. g. Casper (1930—1937), Boor & Miller (1934—1944), Stokinger, Ackerman & Carpentier (1944), Uroma (1943). Uroma contends that he has been able, by means of a specific polysaccharide, to establish four well-defined serological types of gonococcus, whereas Stokinger, Ackerman & Carpenter are unable to demonstrate any specific polysaccharide, and are rather inclined to consider nucleo-protein the most important cellular element. However, they found no type specific component. Apart from the still unconfirmed results obtained by Uroma, the previous investigations have shown that the question of classification of the gonococci into types is still an open one.

In May 1940, at the Danish State Serum Institute, I started to work out a practical method for typing gonococcal strains by means of immune sera. In spite of previous rather discouraging experiences, I still hoped it might prove possible, in this way, to arrive at a positive result. At that time it was easy to obtain many newly-isolated gonococcal strains, as Reynmann (1941) had just systematised the cultivation of gonococci in this country as a diagnostic method.

According to Casper, the preparation of specific polysaccharides appeared to be very circumstantial and to require a large amount of growth. Furthermore, by this method about 40 % of the strains could not be classified. It seems preferable, therefore, to use a more direct method, so that the strains in question are handled as little as possible.

In spite of the employment of quite recently-isolated cultures, this method was found to involve many practical difficulties. Among other things, war conditions made it hard to obtain the agar required. Under such conditions, it was difficult to keep up the continuity of the experiments.

Furthermore, while this work was going on, we had practically no access to foreign literature. Unfortunately, we were also unable to obtain any strains from other countries. (For a literary review, see Alice Reyn. 1947).

1. Definition of the Material.

The present studies cover about 700 gonococcal strains collected from May 1940 to February 1944, and isolated on modified McLeod medium (with addition of about 10 % ascites fluid (Reymann, 1941)). The bacteria met the following requirements: Gram-negative diplococci, characteristic »coffee-bean« shape, isolated from the urethra cervix or vagina of patients suspect for gonorrhea. Further, all the strains, with the exception of 43 atypical ones, were capable of fermenting glucose but not maltose or laevulose.

The strains were stored in two ways:

- a. In semi-fluid 0.2 % broth agar covered by liquid paraffin; transferred every 3—6 months; stored in incubator at 37°.
- b. Ascites-broth culture; dried by cold-vacuum after Fløsdorf & Mudd (1935, 1938); stored in refrigerator at 4°.

Both methods result in losses in the form of dead cultures, but the drying method offers the advantage of being free from transfers of the cultures, thus avoiding the possibility of contamination and mistakes in the labelling of the tubes. In addition, it saves a good deal of work, culture medium and space in the incubator. Finally, it is also practical for mailing purposes. The drying method thus appears to be excellent, and the capacity for growth of such cultures has been lost only in about 10 % of the ampules, even after storage for more than 3 years.

There is no essential difference in the influence of the two methods on the bacteria — neither serologically, nor with regard to the chemo-resistance of the strains in vitro.

As has also been pointed out by other investigators, the chemo-resistance may be increased by growing the strains on media containing sulphathiazol. The antigenic structure of the strains is independent of the degree of chemo-resistance, the original as well as the artificially-increased.

2. Serological Technique.

The complement-fixation test employed for the serological examination was carried out with the technique given by Martin Kristensen (1930).

a. *Serum* — As a rule, rabbits (weighing about 2 kg.) were immunised with formalin-killed gonococci (suspended in buffer saline, pH 7.38, with the addition of 0.5 % formalin, incubated for three hours at 37°).

The first dose was 1 cc. of a suspension containing 1000 million bacteria per cc. = Standard 8 (Wulff, 1921), while the second and following doses were 2 cc. of the same suspension, all injected intravenously.

Standard 8 corresponds to about 1000 million bacteria if the suspension is made up of *E. coli* or *Staph. aureus*. In work with other bacteria, the figure may be corrected by means of the correction values recorded by Cunningham & Timothy (1924). For gonococci and meningococci, the figure has to be

multiplied by 0.95 and 1.40, respectively. In the present work, however, the figure has not been corrected, but I have estimated that Standard 8 = 1000 million bacteria per cc.

After 2 weekly injections had been given for 3—4 weeks, heart puncture was performed, and then the immunisation was continued for the same length of time, again followed by heart puncture. In some cases 3 or 4 portions of serum were obtained from one rabbit, while other rabbits died at the first heart puncture. If a smaller amount of serum was required, the rabbit was bled to death after 4—5 weeks, when a small sample of blood had shown the titre to be suitable. As a rule, a titre of 10—12 degrees of potency was obtained. (With regard to these degrees, see the description of the complement-fixation test given below).

At first, one or two rabbits were immunised with the same strain. Later on, 2—4 animals were used per strain so as to eliminate differences in titre due to the varying capacity of the rabbits for antibody production (cf. Kauffmann (1940) and Erna Morch (1943)).

Serum was inactivated for 30 minutes at 56° and stored (without addition of any disinfectant) in frozen state at $+8^{\circ}$ or for shorter periods at $+4^{\circ}$. Generally it was found that the original titre could be kept in this way for several years.

Several normal rabbits showed a positive gono-reaction (GR), on which account all rabbits were examined with a view to this possibility prior to the immunisation, so as to exclude the »spontaneous« positive reactors. The positive gono-reaction of normal rabbits will be dealt with below (Section 4).

Titration of the serum was carried out as follows: 1st tube, 0.025 cc. undiluted serum; 2nd tube, 0.025/3 cc. serum; 3rd tube, 0.025/9 cc., and so on. For practical reasons, 0.025/3 cc. and 0.025/9 cc. are transferred to the tubes as follows: — 0.05 cc. undiluted serum is mixed in a mixing tube with 0.31 cc. of 0.9 % saline, and 0.06 and 0.02 cc. respectively of this mixture are placed into tubes No. 2 and 3.

b. *Antigen* — An 18—20 hour culture is suspended in 0.9 % saline to a density of 20000 million bacteria per cc. The bacteria are killed by heating to 56° for 20 minutes on water-bath, and then the antigen is stored in refrigerator at $+8^{\circ}$ without addition of any disinfectant. Culture medium — thick broth agar plates with addition of 33 % human ascites fluid. The antigen is adjusted to the optimal dose for homologous (or heterologous) anti-gonococcal sera. Dose employed — 0.30 cc. diluted antigen with addition of complement corresponding to 1 haemolytic unit. The antigen is diluted in a buffer solution pH 7.38.

The experiments showed that for some (? most) antigens there was an optimal zone, within which the fixing capacity was maximal. Other antigens could be used only within a very narrow range, as a rule in a dose which was just not anti-complementary.

As a rule, about 50 million bacteria per tube will give maximal fixation, but such a dose will be anti-complementary in about 20 % of the cases.

The dose of antigen had to be increased more in relation to the dose of complement if the same percentage of haemolysis was to be obtained.

Employment of an individual complement dose in the strongly anti-complementary antigens made it possible to use the latter in higher concentration at the expense of the fixing capacity. At the same time, the optimum of the antigen became »broader«, and this again decreased the risk of the anti-complementary tendency because of errors in measuring the antigen.

Antigens prepared from young cultures were not so anti-complementary as antigens made from older cultures.

Addition of undiluted inactive guinea-pig serum to the complement-fixing

reduced the anti-complementary tendency, but also impaired the fixing capacity.

c. *Blood Cells* — 2.5 % suspension of red sheep cells in equal parts of saline and buffer saline with pH 7.38.

d. *Amboceptor* — Serum from guinea-pigs immunised with kidney emulsion from guinea-pig. Dose employed here — $3 \times$ haemolytic dose as determined by daily amboceptor titration.

e. *Complement* — Fresh guinea-pig serum, «titre» determined daily, corresponding to 1 haemolytic unit in the presence of antigen in the test tube.

In practice, the test is performed as follows:

First, the serum is titrated in 4 or 5 tubes. Then an antigen dilution is made up in buffer saline — for instance, 0.10 cc. concentrated antigen (20000 bacteria per cc.) is added to 15 cc. buffer saline (this amount corresponds to 50 test tubes: $0.30 \text{ cc.} \times 50 = 15 \text{ cc.}$). Complement is added to the mixture, and then 0.30 cc. of the antigen-complement dilution is transferred to each tube (40 million bacteria) by means of an automatic syringe. Then the tubes are shaken thoroughly. The tubes are then left standing at room temperature for 45 minutes, then at 37° for 45 minutes. To each tube is added 0.20 cc. of a suspension of blood cells sensitised as follows: —

To a 5 % stock suspension of washed sheep cells in buffer saline is added an equal volume of saline containing $3 \times$ haemolytic dose of amboceptor. $0.20 \times 50 = 10 \text{ cc.}$ of this suspension is necessary for 50 tubes.

After shaking, the test tubes are left standing on water-bath at 37° for 1 hour. The tubes are stored in the refrigerator overnight.

On the following day the percentages of haemolysis are read, the values obtained being compared with a scale of haemolysis prepared as follows: A few cc. of clear red fluid are collected from tubes with complete haemolysis (for instance, serum controls). Quantities of 0.50, 0.40, 0.30, 0.20 and 0.10 cc. of this fluid are placed in tubes of the same size as employed in the test (Wassermann tubes), and the volume is made up to 0.50 cc. in all the tubes by means of distilled water or saline. This gives a colour scale with 100, 80, 60, 40 and 20 % haemolysis, from which the percentages of haemolysis in the test tubes can be read by direct comparison.

Volume of contents of each test tubes:

Serum: 0.025 cc. undiluted — diluted 006 at the most.

Antigen: 0.30 cc. antigen diluted in buffer saline containing complement corresponding to the «titre».

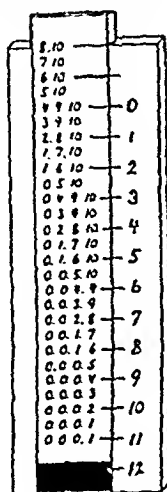
Red Cells: 0.20 cc. with addition of $3 \times$ amboceptor titre.

Total: about 0.50 cc.

From the percentages of haemolysis read on the following day, the degree of potency of the reaction is calculated after the method given by Martin Kristensen (1930), the degree being defined as the value for n in the formula $a = 0.025 \times 3^{-n}$ required to make a exactly the volume of serum containing just enough antibody to give a minimum reaction, i.e. 60 % haemolysis. This holds good when the experiment is adjusted «normally». Since this is hardly ever the case, a correction based on control sera with known values (see below) may be employed.

The haemolysis percentages are converted into degrees of potency by means of a «sliding rule», on which the haemolysis percentages corresponding to the more common results are entered on a movable scale, while the corresponding degrees of potency are given on the fixed scale. N.B.: — For

practical reasons, 0, 10, 20 100 % haemolysis are entered on the scale as 0, 1, 2 10 (see the following drawing): —



If all the complement-fixation tests are carried out with several *control sera of known potency*, it is practicable, on the average deviation from the standard values, to correct the experimental values obtained by means of the movable scale. If, for instance, the average deviation of the control sera is $+1^{\circ}$ (*), the scale is adjusted so that the haemolysis percentage ($40 \approx 4$), which in «normal» tests without deviation stands at the level of 1° on the fixed scale, now stands at the level 0° .

Variation in the results of complement-fixation tests may be due to several causes; primarily to variations in the preparation of the complement-antigen mixture and the haemolytic system, and further, to accidental errors in the titration of the serum and to the addition of antigen and haemolytic system with automatic syringe. The systematic variations may be adjusted by employment of control sera of known potency that are titrated together with the other sera in the same experiment. Using control sera in connection with correction of his haemolytic scale, Martin Kristensen estimates the «mean error» to be $\frac{1}{2}$ — 1° .

In the present work, control sera have not been employed systematically. Instead, an attempt has been made to eliminate the experimental variation by taking all the reactions to be compared as far as possible on one day. The «daily capacity» was about 1000 test tubes.

It has not been possible to make corrections for accidental errors. However, exclusion of such errors has been ensured by repetition of the test in cases where the result differed from what was expected, or in sudden pronounced deviations from the results of tests with gradual changes in various components.

Furthermore, in cases with results deviating from those of previous tests, allowance has been made for correction on the basis of control sera using during the taking of ordinary gono-reactions in the Serum Diagnostic Department.

Finally, various control tests were made continually, viz: — the ability of the serum to prevent the complement from acting without addition of antigen = anti-complementary effect of the serum; and the same ability of

(*) Degrees of potency.

of the antigen without addition of serum = anti-complementary effect on the antigen. In the following, these two kinds of control will be designated as *serum control* and *antigen control* respectively.

From preliminary complement-fixation experiment with 10 gonococcus strains picked out at random and the corresponding immune sera, it was evident (as expected) that the direct complement-fixation reaction was insufficient for typing of gonococci, on which account I went on to making absorption experiments.

3. *Absorption Technique.*

The *absorptive antigen* consisted of a suspension of formalin-killed gonococci in buffer saline with pH 7.38. This antigen was stored at 4°. The density of the suspension was about 20000 million bacteria per cc.

The *serum* was inactivated rabbit serum, diluted 1:10 with saline (for practical reasons) or, in the later experiments, undiluted guinea-pig serum.

After centrifuging the absorptive antigen (3000—5000 r. p. m. for 1 hour), either in ordinary Wassermann tubes (about 1.5 × 6 cm) or in tall tubes of the same diameter (about 1.5 × 10 cm), the supernatant fluid was pipetted off with a Pasteur pipette. Any remnants of fluid on the sides of the tubes or just above the sediment were removed with a strip of filter paper, so that in the process of absorption the serum could not be diluted to any particular degree.

The serum dilution (as a rule, 1.5—2 cc.) was placed over the sediment, which was suspended carefully. The mixture was then allowed to stand at 4° until the next day. The absorption could just as well have been carried out by leaving the mixture at 37° for 30 minutes, but for practical reasons the other procedure was employed.

After centrifuging as mentioned above, the diluted serum was withdrawn carefully with a Pasteur pipette.

The *absorption dose* was determined in some preliminary experiments. As a rule, it was 6—8—10 cc. antigen (120000—20000 million bacteria) per 10 cc. of serum dilution. After the absorption, the serum dilution was titrated as follows: 1st tube, 0.075 cc.; 2nd tube, 0.025 cc.; 3rd tube, 0.25/3 cc., and so on.

In this work, »emptying« of a serum means that the complement-fixing antigen corresponding to the absorptive antigen does not react with the absorbed serum, whereas »complete absorption« means that neither the heterologous (absorbent) nor the homologous (immunising) antigen reacts with the absorbed serum in complement-fixation tests.

As far as possible, the immunising, complement-fixing, and absorptive antigen preparations were prepared at the same time.

In *control tests* with absorption of anti-gonococcal sera with entirely different bacteria (e. g. Gram-negative rods such as paratyphoid B and Salmonella Newport), at the most 1—2 degrees of potency were removed.

Cross-absorption experiments with 7 strains and their corresponding sera showed the presence of at least 6 partial antigens, viz. one antigen common to all gonococci (species antigen), which for the present will be designated as *a*, and at least 5 partial antigens that are strain- or type-specific.

The designation of the antigens was carried out after pairwise consideration of antigens and sera, as illustrated by the following example:

	Complement fixation with antigen:			Complement fixation with antigen:	
	x	y		y	x
Unabsorbed Serum X	++	++	Unabsorbed Serum Y	++	++
Absorbed with antigen x	—	—	Absorbed with antigen y	—	—
‘ ‘ ‘ y	+	—	‘ ‘ ‘ x	+	—

In this example, strains *x* and *y* have antigen *a* in common, as both the corresponding sera react with both antigens. However, in addition, *x* must possess a partial antigen *b*, which *y* is not able to remove from Serum X, while *y* must possess a partial antigen *c*, which *x* cannot remove from serum Y. Thus, the *antigen* components are designated with small letters, the corresponding *antibody* components with capitals.

Unfortunately, the *absorbed sera were often anti-complementary*, making it sometimes impossible to interpret the results properly. On this account, various attempts were made to counteract this tendency. Centrifuging for 1 hour at 15000 r.p.m. was found not to counteract the anti-complementary tendency, and the same applies to absorption of the serum in active state and inactivation of the serum after absorption.

Preliminary treatment of the absorptive antigen with *inactive guinea-pig serum abolished or lowered the anti-complementary tendency* of absorbed sera, but at the same time *some of the fixations were weakened*. The proportion of absorptive antigen to guinea-pig serum was 1—2 or 1—1. The homologous fixations were preserved *almost unchanged*.

Preliminary treatment with *buffer saline*, with or without addition of formalin, counteracted the anti-complementary tendency in some cases, but as a rule it was not as effective as inactive guinea-pig serum.

»After-washing« with buffer saline after preliminary treatment with guinea-pig serum, would sometimes cause the anti-complementary tendency to reappear.

Addition of active or inactive guinea-pig serum to the diluted serum that was to be absorbed, gave a slight decrease in the anti-complementary tendency of the serum.

Employment of *young cultures* as absorptive antigen did not prevent the absorbed serum from becoming anti-complementary.

Trypsin-treated absorptive antigen gave rise to a much stronger anti-complementary tendency than antigen prepared in the usual manner.

Further, the course of the absorption in homologous and heterologous sera was investigated, as well as the relation between the absorption dose and the degrees of potency removed in various sera. The result was as follows:

Homologous absorption gave an abrupt decrease in potency, irrespective of the antigen employed for the reaction.

Heterologous absorption gave a fairly abrupt decrease in potency as measured with the absorptive antigen, but a less abrupt decrease as measured with the homologous antigen, and probably, as a rule, a constant »remaining reaction«.

In some sera it was possible, by heterologous absorption, to obtain a gradual decrease in the homologous remaining reaction, in spite of reaction being lacking with the absorptive antigen in lower absorption doses.

Generally speaking, the absorption appears to proceed just as easily in strong sera as in weak ones.

4. *Positive Gono-reaction in »Normal« Rabbits.*

As mentioned above, serum from non-immunised, apparently normal rabbits was often found to cause reaction with gonococcal antigen. This phenomenon has been mentioned previously by a number of authors (e. g. Torrey, Watabiki, Pearce, Tulloch, Vollmond, Segawa, Cohn, Lentz & Schäfer, and Casper), but so far the question has not been elucidated thoroughly. Stokinger, Carpenter & Plack (1944) mentioned that normal rabbit serum dissolves certain cellular components of the gonococcus; this may perhaps be interpreted as an antigen-antibody reaction, but is probably due to autolysis.

It was found that normal rabbit serum gave as strong a reaction with newly-isolated strains as with older ones (10 fresh and numerous older strains were tested) — cf. Tulloch (1922), who claimed that it was particularly rabbit sera with older cultures that gave strong reactions.

Altogether about 1000 rabbit sera were examined because, as mentioned, only rabbits giving a negative gono-reaction were used for immunisation; and only about 30 % of the adult rabbits examined gave a negative reaction.

The age of the animals was found to play a rôle in the appearance of the positive gono-reaction. About 90 % of the quite young animals (7—8 weeks old) showed a negative gono-reaction, whereas a negative reaction was seen in only 42 % of the older rabbits (4—5 months old) (altogether 90 rabbits were examined for determining this particular feature).

Probably this difference between younger and older rabbits signifies immunity to some kind of infection, past or present.

Several experiments showed that this *positive reaction does not signify any tendency to »non-specific« complement-fixation*. Thus, for

instance, a positive Wassermannreaction, which may occur in »normal« animals is not parallel with positive gono-reaction, and the positive gono-reaction can be removed by absorption with gonococci without simultaneous abolition of the positive Wassermannreaction (»Wassermann antigen«, Mörch 1933).

Furthermore, experiments were carried out to ascertain whether the antibody giving the positive gono-reaction is always the same (perhaps corresponding to antigen α common to all gonococci), or whether several partial antibodies are involved. Direct complement-fixation tests indicated that it was undoubtedly the same antibody that reacted in all these cases. Eight normal rabbit sera were tested with 8 serologically different gonococcus strains, and the reactions of the various sera with the different strains were surprisingly uniform.

In order to investigate further into this question, a normal rabbit serum (with positive gono-reaction) was observed with a number of different bacterial antigens, viz.: in addition to two different gonococcus strains, a haemolytic streptococcus (Group A, type 17), a strain of micrococcus catarrhalis, and an accidental strain of Staph. aureus. Only the gonococcal antigens were able to empty the serum completely. Like the direct complement-fixation test, this result indicates that the positive reaction is due to an antigen common to all gonococci.

M. catarrhalis, streptococcus, staphylococcus and pneumococcus (Type 1) antigens were able to remove some of the reaction. This was not surprising as far as m. catarrhalis antigen is concerned, as antigenic relationship between gonococcus and m. catarrhalis has been demonstrated previously (e. g. Reyn, 1943). As to the pneumococci, Boor & Miller (1931, 1934) found a certain relationship between these bacteria and the gonococci, as nucleo-protein prepared from gonococci reacted with anti-pneumococcal sera corresponding to Types 1, 2 and 3, while polysaccharide prepared from gonococci reacted with anti-pneumococcal serum corresponding to Type 3, and also with serum corresponding to rough pneumococci.

The results obtained with the streptococci and staphylococci are more difficult to explain, especially as these strains did not react with serum in direct complement-fixation test.

Summarising these findings, it may be said that *practically all gonococcus strains are able to remove the »false« positive gono-reactions from normal rabbit sera, whereas a number of other bacterial antigens are incapable of doing so.*

The consequence of these experiments must be that in immunisation of rabbits there is no risk of coming across »non-specific« antibodies aimed against gonococcal antigens other than the common antigen α . However, while this part of the work was going on, I was afraid for a while that perhaps non-specific antibodies might be produced in rabbits during immunisation with gonococcus antigen, even though the gono-reaction was negative at the commencement of the

immunisation. Such antibodies might conceivably interfere with the establishment of antigenic formulas unless they corresponded to the antigen *a*, common to all gonococci. If so, all gonococcus strains would be able to remove the antihodies, which would then play no rôle in this connection.

This was one of the reasons why I went on to use guinea-pig sera instead of rabbit sera (for details, see the next paper).

The next step was to investigate some of the bacteria which are known from experience to be found in rabbits showing no sign of disease, as this seemed to be the easiest way of securing the bacterium or bacteria that might be the cause of a positive gono-reaction in »normal« rabbits.

One micro-organism of this category is *B. septicaemiae*, *haemorrhagicae* (Pasteurella), which several workers state may be isolated from the nose and throat of rabbits apparently perfectly healthy or having only slight coryza.

Hanger (1927) found that Pasteurellae could always be isolated from rabbits with coryza. He examined the cutaneous allergy in experimental rabbits among which infection with Pasteurellae appeared endemically. Most of the animals gave allergic skin reactions to intracutaneous injection of Berkefeld filtrate of Pasteurella broth culture — which was indeed not strange. On the other hand, it was striking that filtrates from *colon*, *influenza* and *meningococcus* cultures produced allergic reactions in the same rabbits. Guinea-pigs gave irregular or negative reactions, and rabbits younger than 3 weeks gave negative reactions.

Morch & Krogh-Lund (1931) also found antibodies (agglutinin) against Pasteurella in serum from apparently normal rabbits.

Miller & Castles (1933) examined the skin allergy in rabbits given agar foci containing gonococci (6 strains), meningococci (2 strains), *m. catarrhalis* (1 strain), and *Pastenrella* (1 strain supplied by Webster) respectively. The authors found overlapping allergy in animals with foci of gonococci and meningococci, and also, to some extent, with *m. catarrhalis*, but no cross reactions in Pasteurellae or any of the remaining 3 bacterial species. These experiments were carried out on rabbits free from coryza.

In the following, descriptions of serological and bacteriological studies on the relationship between Pasteurellae and gonococci will be given.

5. Serological Relationship between Gonococci and Pastenrellae.

a. Serological Studies on Laboratory Strains.

On examination of a number of »normal« rabbit sera and guinea-pig sera in complement-fixation tests with 8 different Pasteurella antigens prepared from 8 Pasteurella strains isolated at the State

Serum Institute, Copenhagen, a high percentage of the rabbit sera were positive. The technique was the same as described above for the gonococci.

The various *Pasteurella* strains are designated as P. I, P. II P. VIII. Of these strains, P. I and P. IV showed the highest percentages of positive reactions with rabbit sera — 62 % and 75 % respectively. As was to be expected, the percentage of positive gono-reactions was also high — 42.3 %.

Just as with the *“false”* gono-reactions, considerably more positive reactions — for instance, to P. IV — were obtained in the older animals than in the younger ones, as illustrated by the followings: —

No. of animals	Age	P. IV R.: positive	P. IV R.: doubtful	P. IV R.: doubtful	P. IV R.: negative
25	7–8 weeks	approx. 1000 gr.	19 (aver. 2.7 °)	0	6 (24 %)
30	11–12 „	1400–1600 „	13	0	17 (57 %)
25	12–14 „	1600–1800 „	17	6	2 (8 %)
50	approx. 5 months	2500 „	48 (aver. 3.6 °)	0	2 (4 %)
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In addition, there was a *distinct correlation between positive gono-reaction and positive P. IV R.* (and P. I R), except that the *Pasteurella* reactions were stronger than the gono-reactions.

A number of immune sera (anti-pasteurellar and anti-gonococcal) were then tested with both *Pasteurella* and gonococcus antigens. Both guinea-pigs and rabbits were immunised with the 8 *Pasteurella* strains — altogether 78 guinea-pigs and 13 rabbits. Several sera from animals immunised with P. I, P. III, P. IV and, to a lesser degree, P. V and P. VI, reacted with gonococcus antigen 12954 (*“Type”* $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} & \text{XII} \\ + & - & + & - & - \end{matrix}$, see the following paper). These overlapping reactions were particularly evident with P. IV sera, and all these sera (15) gave fixation with the gonococcal antigen, just as the ordinary gono-reaction (with polyvalent gonococcal antigen) was also positive. This corresponded to the fact that P. IV, which showed the highest reaction percentage among *“normal”* rabbits, was the strain found to have most in common with gonococci.

Then *anti-gonococcal sera* were tested with *Pasteurella* antigens. First 39 sera from guinea-pigs immunised with different gonococcal strains were tested by complement-fixation test with P. V, with negative result. Then the other strains were employed in tests both with guinea-pig and rabbit sera.

¹⁾ P. IV R. = complement-fixation with P. IV.

²⁾ The figures in brackets give the degrees of potency.

On account of the varying tendency of the *Pasteurella* antigens to react with serum from «normal» animals, complement-fixation with guinea-pig and rabbit sera will be mentioned separately.

P. I, P. II, P. III and P. IV reacted rather strongly in the 5 guinea-pig sera examined, whereas P. V, P. VI, P. VII and P. III reacted only weakly or not at all. This means that *gonococci and certain Pasteurella strains are serologically related*.

Rabbit sera also gave a number of positive reactions, but these are not as convincing as the results obtained with guinea-pig sera, because «normal» rabbit sera, for instance, give a positive P. IV reaction in 75 % of the cases examined.

Immunisation with *Salmonella Newport*, paratyphoid A and B (6 guinea-pigs per antigen) did not produce antibodies against *Pasteurella* or gonococci. There was one exception to this rule, however, as the serum from one animal immunised with paratyphoid B proved to react very strongly both with gonococcal antigen and with *Pasteurella* an-

Table 1.

Cross-absorption Experiment with Gonococci and Pasteurellae in Guinea-pig and Rabbit Sera.

(Experiment performed on 11th January, 1945.)

Sera absorbed on 10/1—11/1 1945.

Values recorded = degrees of potency.

Native guinea-pig serum/12954: serum from guinea-pig immunised with gonococcus strain 12954.

1. <i>Gonococcus</i> immune sera:	Complement-fixation with:		
	Gonococcus (homologus)	<i>Pasteurella</i> No. IV	Serum control
Non-abs. guinea-pig serum/12954	10½	10½	0
» rabbit serum/37496	15	8½	0
Guinea-pig serum/12954 absorbed homologously	0	0?	0
Rabbit serum/37496 absorbed homolog. .	0	0	0
Guinea-pig serum/12954 abs. with Past. No. IV	8	0	0
Rabbit serum/37496 abs. with Past. No. IV	12	0	0
2. <i>Pasteurella</i> immune sera:	Gonococcus 11391	<i>Pasteurella</i> No. IV (homologus)	Serum control
Non-abs. guinea-pig serum/Past. No. IV	8½	9	0
» rabbit serum/Past. No. IV	8½	≥ 12	0
Guinea-pig serum/Past. No. IV abs. with Gon. 11394	0	6	0
Rabbit serum/Past. No. IV abs. with Gon. 11394	0	8½	0
Guinea-pig serum absorbed homolog. .	0	0	0
Rabbit serum/Past. No. IV absorbed homologously	½	0	0

tigen XII (see below). Unfortunately, this animal was destroyed before the serum was examined, so that no bacteriological examination could be carried out.

Since it had now been demonstrated by direct complement-fixation that possibly there was a serological relationship between certain *Pasteurella* and gonococci, *absorption* experiments were carried out. As it was impracticable to investigate all the *Pasteurella* strains in this way, one — viz. P. IV — was selected for these experiments. Table 1 gives the results of some absorption experiments with gonococci and P. IV, together with the corresponding sera, both with rabbit and guinea-pig sera.

In direct complement-fixation tests, the anti-gonococcal and anti-pasteurellar sera reacted with both antigens; the homologous titres were higher, but the heterologous were also fairly high.

Assuming that there was no antigenic relationship between the two bacterial species, it would seem conceivable that the reaction P. IV with anti-gonococcal serum was due to an accidental infection of the animal with P. IV during the immunisation with gonococci. If so, the direct complement-fixation test would show a reaction of the serum

Table 2.

Cross-absorption Experiment with Gonococcus Strain 12954, Pasteurella IV and m. catarrhalis 465 in the corresponding Rabbit Sera.

(Experiment performed on 16th January, 1945.)

Sera absorbed on 15/1—16/1 1945.

Values recorded = degrees of potency.

	Complement-fixation with:				
	Gonococcus (homolog.)	Past. No. IV	Micrococ. cat.	Salmonella Newport	Serum control
Non-abs rabbit serum/Gon. 12954	15	13	0	0	0
Abs. homologously	0	0	0	0	0
Absorbed with Past. No. IV	9	0	0	0	0
» » m. cat. 374	12	10	0	0	0
» » Salm. Newport ..	15	12	0	0	0
	Complement-fixation with:				
	Gonococcus No. 12954	Past. No. IV (homolog.)	Micrococ. cat.	Salmonella Newport	Serum control
Non-abs. rabbit serum/Past. No. IV	9½	12	0	0	0
Absorbed with Gon. 12954	0	6	0	0	0
» homologously	0	0	0	0	0
» with m. cat. 374	0	9	0	0	0
» » Salm. Newport ...	9	11½	0	0	0
	Complement-fixation with:				
	Gonococcus No. 12954	Past. No. IV	M. cat. (homolog.)	Salmonella Newport	Serum control
Non-abs. rabbit serum/m. cat. 465	0?	0	9	0	0
Absorbed with Gon. 12954	0	0	8½	0	0
» » Past. No. IV	0	0	8½	0	0
» homologously	0	0	0	0	0
» with Salm. Newport ...	0	0	8½	0	0

with gonococcal antigen and P. IV antigen, while absorption with gonococcal antigen would remove only the fixation with gonococcal antigen, and the P. IV reaction would remain.

As a matter of fact, both the *homologous and the heterologous reactions* were removed by homologous absorption from the anti-gonococcal sera as well as from the anti-pasteurellar sera — and this is indicative of *antigenic relationship*.

Heterologous absorption removed only a small part (about 3 degrees of potency) of the homologous reaction of the sera. Thus, besides their common antigen, there must be *other antigenic quota*, specific to gonococci and P. IV respectively.

As some relationship had been demonstrated previously between gonococci and certain strains of *m. catarrhalis* (e.g. Reyn, 1943), it seemed obvious to look into the possible relation between *m. catarrhalis* and P. IV. Table 2 gives the results of cross-absorption experiments with the following strains and the corresponding rabbit immune sera: gonococcal strain 12954, P. IV and *m. catarrhalis*. For control, the sera were also absorbed with *S. Newport*.

N. B.: The *m. catarrhalis* anti-serum was produced by immunisation with *m. catarrhalis* No. 455. As mentioned before, however, this strain had died, on which account another selected strain, No. 374, had to be used for the absorption.

This experiment showed that gonococcus strain 12954 and P. IV presented greater serological relationship than between *m. catarrhalis* and strain 12954 and P. IV respectively.

In direct complement-fixation tests, the gonococcal strain and P. IV reacted with a high titre in each other's sera, whereas *m. catarrhalis* gave fixation only with the *m. catarrhalis* anti-serum, which in return reacted neither with gonococcal strain 12954 nor with P. IV. The serological relationship between gonococcal strain 12954 and P. IV was confirmed by the absorptions, and it is only reasonable to assume that the sera examined contained abundant antibody corresponding to the common antigen, as the homologous reaction on heterologous absorption decreased by about 6 degrees of potency in both sera.

In spite of there being no reactivity in direct complement-fixation tests, *m. catarrhalis* removed up to 3 degrees of potency of the gono-reaction and the P. IV reaction both from serum 12954 and serum P. IV, while absorption with *S. Newport* made practically no difference to the reactions.

The *m. catarrhalis* serum could be emptied only of *m. catarrhalis* antigen, while gonococcus strain 12954, P. IV and *S. Newport* left the fixation with *m. catarrhalis* antigen unchanged after absorption.

The antigenic formulas of the 3 bacteria examined might, for instance, be illustrated as follows: —

Gonococcal strain 12954:	$a_1 a_2 x$
P. IV:	$a_1 a_2 y$
M. catarrhalis	$a_2 z$

in which a_1 is bigger than a_2 .

In the preceding, the mutual gonococcal antigen is designated as a . Probably it would be more reasonable to assume the serological relationship between the three bacterial strains to be limited to antigen a , which might possibly consist of some quota such as, for instance, a_1 and a_2 , while x , y and z represent the antigen quota specific to the respective species.

Gonococcal strains 12954 and 11394 were not the only gonococci strains capable of complement-fixation in a number of Pasteurella sera in direct tests. Several other strains of different »types« (see the following paper) were examined, and proved to react in the same way — which was to be expected if the antigenic relationship was due to a , $a_1 a_2$ respectively.

In addition, 5 anti-pasteurellar sera (corresponding to P. IV—P. V) were absorbed with 3 gonococcal strains of different serological »type« and with m. catarrhalis 374. The four antigens removed from 3—6 degrees of potency of the homologous reaction of the sera, even though the corresponding antigens in direct complement-fixation tests have, in some instances, shown negative reactions.

In order to ascertain whether the serological relationship between gonococci and Pasteurellae were solely due to the mutual gonococcal antigen(s), or whether other partial gonococcal antigens might be found in Pasteurellae, antigen prepared from P. I—P. VIII was tested with factor sera (see the following paper), homologous anti-pasteurellar serum and 2 anti-gonococcal sera corresponding to strains 12954 and 2 e (used in the production of factor sera III and IX). The antigen was examined after heating to 56° and 100° (cf. experiments of similar character with gonococcal antigens to be reported in a subsequent paper).

P. IV reacted (3—5 degrees of potency) with factor serum VI, corresponding to »antigen 56° « and »antigen 100° «. P. IV and P. VII gave a weak reaction (about 2 degrees of potency) with factor serum XII, but only corresponding to »antigen 100° «.

As a matter of fact, practically all the homologous reactions were about 3 degrees of potency stronger with »antigen 100° « than with »antigen 56° «, just as in several cases the anti-gonococcal sera gave a negative reaction with »antigen 56° « and a positive reaction (about 2 degrees of potency) with »antigen 100° «.

Thus, the experiment shows that *gonococci* and *Pasteurellae* occasionally have some partial antigens in common.

Direct complement-fixation tests with gonococci and Pasteurellae together with their corresponding sera (various strains), performed with antigen heated to 56° and 100°, showed that the overlapping reactions were strongest with »antigen 100°«. In this connection, it may be mentioned that Jessen (1933) states that the results from a single complement-fixation test with »boiled« meningococci and gonococci were more »non-specific« than the results obtained with the same antigens heated to 60°. Similar results were obtained by Miller and collaborators (1943) with heat-killed meningococci.

P. I—P. V and the corresponding guinea-pig sera were examined by direct complement-fixation tests, each serum being tested with 5 antigens. In this way, the homologous were always found to be stronger than the heterologous reactions, which in some cases were entirely negative. Cross-absorption experiments showed no identity between any of the 5 strains — which indeed seemed most probable after the direct complement-fixation tests.

Without entering further into the antigenic structure of these strains, it should be mentioned that *P. I* and *P. IV* were closely related, and that *P. II* occupied a position by itself, as the *P. II* serum reacted practically only with the homologous antigen; in addition, the homologous reaction of this serum could only be removed by homologous reaction.

Previous experiments had shown that many *P. I* and *P. IV* sera, but no *P. II* sera, reacted with gonococcal antigen.

Conclusion: Normal rabbit sera often react with certain Pasteurella strains, and the positive reaction is correlated with positive gonoreaction.

Direct complement-fixation and cross-absorption experiments show that at any rate certain Pasteurella strains and gonococci are closely related. This serological relationship is chiefly due to the fact that the antigen common to all gonococci occurs also in certain Pasteurella strains.

Some Pasteurella strains and certain gonococci have partial antigens in common, other than the antigen common to all gonococci.

As a rule, the overlapping reactions are stronger with antigen heated to 100° than with antigen heated to 56°.

All the Pasteurella strains examined were different. However, the two strains most closely related to the gonococci were also closely related to each other.

b. Bacteriological Studies on Pasteurella Stock Strains and Strains isolated from Rabbits at the State Serum Institute.

The next task was to isolate Pasteurella from our stock of rabbits. These experiments were carried out between December 1944 and February 1945. At first, nasal cultures were made from the rabbits by means of an ordinary cotton swab, without preliminary treatment of the nasal mucosa. No Pasteurellae were isolated in this way. On the other hand, some strains of *m. catarrhalis* were obtained that reacted neither with homologous serum nor with gonococcal serum.

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Meyer (1928) has recommended instilling one drop of 1 % silver nitrate solution in each nostril and taking cultures after 3 days. He claims that this method offers better possibilities of finding *Pasteurellae*.

However, even using Meyer's technique, I did not succeed in isolating any *Pasteurella*-like bacteria until I used a thin, flexible, twisted wire for collecting material from the nasal cavity. After wiping the vestibule of the nose with an ordinary swab, the thin wire was passed several centimeters up into the nose, gentle pressure being required to overcome a sphincter-like resistance at the entrance to the nasal cavity proper.

7 *Pasteurella*-like strains were obtained from a total of 33 adult rabbits (almost in pure culture) (2 »strains« were obtained from one rabbit). Macroscopically and microscopically, these strains resembled *Pasteurellae*. The cultures had a characteristic, somewhat-acid odour of the same kind as P. I—P. VIII cultures. Further, on agar the colonies showed a tendency to »grow down in the plate«, which somewhat impeded the preparation of antigen. Blood plates gave a better and easier yield of antigen than did ascites agar plates; for, in spite of abundant growth, the latter gave only a relatively slight yield of antigen — owing to the »down-growth«. No haemolysis was noticed on blood plates. On suspension in 10 %, 5 % and 2.5 % taurocholate, the bacteria were dissolved within 10 minutes.

These bacteria were small, non-motile, Gram-negative rods, which fermented the following sugars without gas production: sorbitol, xylose, maltose, salicin, saccharose, glucose, and mannitol. They were, however, unable to ferment adonit, dulcitol, arabinose, rhamnose and lactose. There was no indole formation even on massive inoculation of the tubes and incubation for several days. In comparison, it may be mentioned that P. IV, which previously had been identified as a *Pasteurella* strain, was also incapable of indole formation in some experiments (March 1945 and May 1945), whereas it showed pronounced indole formation in June 1945, even using the same batch of broth as before.

All the isolated strains were of the same fermentative type. However, the two strains that were isolated from the same rabbit were not quite identical, as it took one of them 3—4 days to show typical fermentations. The colonies of the two strains differed also in appearance, the strains of one colony being strikingly small and mucoid, while the colonies of the other strain were concentrically striped.

Clinically there was no connection, for instance, between the presence of coryza and a positive result of the cultures; some of the animals had a fairly mild degree of coryza but appeared otherwise to be well. Besides coryza, one rabbit had also conjunctivitis, and another rabbit showed ulceration at the nose; these two rabbits (Nos. 6216 and 6219) gave positive cultural results.

Furthermore, 25 young rabbits (weighing about 1000 g.) were examined, a positive result being obtained in one only. The strain here was fermentatively identical with the first strain isolated.

As mentioned above, no indole formation by the strains could be induced in indole broth (casein broth with pH 7.7, diluted with two parts water) (for details, see Kristensen, Lester & Jörgens (1925)). This is rather striking, as most authors consider indole formation an important criterion, even though Lignières (1900), Voges & Proskauer (1898), Busson (1921), Jones (1922), Magnusson (1914) found strains that did not form indole. Mørch & Krogh-Lund (1931) think that this may have been due to the composition of the broth, or that the bacteria mentioned might not have been Pasteurellae. As already mentioned, all the strains were fermentatively identical, showing even fermentation of *salicin*. Mørch & Krogh-Lund (1931) examined 153 Pasteurellae strains of different origin, and none of them was able to ferment this »sugar«. The same has been reported by many other authors (e. g. Kauffman, (1933)), whereas Tanaka (1926) found 16 strains, all of different origin, and all fermenting *salicin*.

In spite of the deviations mentioned, I am of the opinion that it is still justified to regard the strains isolated here as true Pasteurellae.

Conclusion: — From nasal cultures from 33 apparently healthy rabbits, 7 yielded growth of Pasteurella-like bacteria which were identical fermentatively.

c. Serological Studies.

All the Pasteurella-like strains isolated from »normal« rabbits were found to be probably serologically identical also. Further, they reacted only weakly with a number of different anti-pasteurellar sera, and vice versa. This does not indicate that the strains are not true Pasteurellae, as »crossing« of other true Pasteurellae often gives very weak heterologous reaction or none at all.

These Pasteurella-like strains (P. IX—P. XVI) showed a lower reaction percentage with normal rabbit sera than did, for instance, P. IV, and correspondingly only one of the immune sera produced with the new strains (P. IX) gave a weak gono-reaction.

Discussion: Many »normal« rabbits show a positive gono-reaction, and this »spontaneous« gono-reaction is found to be distinctly related to the positive reaction with various Pasteurella antigens, especially P. IV.

Direct complement-fixation tests and cross-absorption experiments show that at any rate certain Pasteurella strains and gonococci are closely related serologically.

A Pasteurella-like organism was isolated from some apparently perfectly healthy rabbits in the stock of the State Serum Institute. This organism was not serologically or fermentatively identical with any

of the *Pasteurella* strains that had shown serological relationship with gonococci. Of 6 guinea-pigs immunised with this strain, only 1 gave a weak reaction with sera from »normal« rabbits that were known to give positive gono-reaction and positive reaction with P. IV.

It is thus reasonable to assume that this strain contained only a slight amount of the antigen common to gonococci and some *Pasteurellae*.

It still seems justified, however, to explain the positive gono-reaction of »normal« rabbits by stating that these rabbits are really *Pasteurella* carriers, and that the positive gono-reaction appears on account of antigenic relationship between *Pasteurellae* and gonococci.

Even though infection with *Pasteurellae* is very rare in man, it may be that here we have one of the causes of otherwise inexplicable positive reactions with human serum and ordinary polyvalent gonococcus antigen.

Summary.

After a brief historical review of demonstration of serological gonococcus types, a definition is given of the material (about 700 strains) investigated by the writer. Mention is made of the methods employed for storage of these strains, viz. drying ad modum Flösdorf & Mudd, and in semifluid agar covered by liquid paraffin.

The serological technique (complement-fixation) is described. From preliminary experiments it is evident that direct complement-fixation is insufficient for typing of the gonococci, on which account the writer goes to the use of absorption.

There is an antigen common to all gonococci (species antigen) besides a number of partial antigens which are type-specific or strain-specific. On absorption, the rabbit sera often become anti-complementary. Various attempts at preventing this anti-complementary tendency are mentioned. The best appears to be preliminary treatment of the absorptive antigen with inactive guinea-pig serum. Further, the course of the absorption in homologous and heterologous sera is investigated, also the relation between the absorption dose and the decrease in the potency of sera differing in strength.

Many sera from apparently normal rabbits react both with polyvalent gonococcal antigen and with more »accidental« gonococcal antigens, whether they are prepared from newly-isolated cultures or from older laboratory strains.

Direct complement-fixation tests and absorption experiments appear to show that the positive gono-reaction is due to an antigen common to all gonococci, as the reaction may be removed completely only by gonococcal antigen. Many normal rabbit sera also react with certain *Pasteurella* strains, and this positive reaction is related to positive gono-reaction. Direct complement-fixation and cross-absorption experiments show that at any rate certain *Pasteurella* strains and go-

nococci are closely related serologically. This serologic relationship is due chiefly to the fact that the antigen common to all gonococci also appears in certain *Pasteurella* strains.

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PENICILLIN-RESISTANT PATHOGENIC STAPHYLOCOCCI ISOLATED FROM THE UPPER RESPIRATORY TRACT

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The clinical aspect of infections with staphylococci has been completely revolutionized since the introduction of penicillin. But although the staphylococcus is within the therapeutic range of penicillin, it is not so penicillin-sensitive as certain other germs, such as the gonococcus, and there are strains which are from the outset penicillin-resistant and others whose resistance is increased *in vitro* and *in vivo*.

We do not at present know how great a part this quality of penicillin-resistance in staphylococci plays in clinical practice. But it may be assumed that certain therapeutic failures can be traced to such a resistance in some strains (1. 2. 3.).

Penicillin-resistant staphylococci occur quite frequently in the natural state. *Spink, Ferris* and *Vivino* (4) found that 12 per cent. of the coagulase-positive strains isolated from morbid processes were resistant. *Rantz* and *Kirby* (5) examined 70 strains from various clinical cases and found 21 per cent. resistant. *Meads* and his colleagues (6) isolated 36 strains from pyogenic processes and found 15 per cent. resistant. *Bondi* and *Dietz* (7) 13.9 per cent., and *Blair, Carr* and *Buchman* (8) found among 79 coagulase-positive strains (isolated for the most part from cases of osteomyelitis) resistance in 21.5 per cent. *Ødegaard* (9) examined 50 strains from lesions of the skin and found as great a proportion as 28 per cent. penicillin-resistant.

While natural resistance seems to be associated with the capacity to produce penicillinase, the resistance which can be provoked *in vitro* is independent of penicillinase formation. This is also a temporary property which soon disappears when the germ is cultivated for a time on penicillin-free media (10).

On the other hand, the resistance which results from treatment with penicillin seems to be a permanent property, and such strains also produce penicillinase. This form of acquired resistance does also not seem to be associated with any coincident loss of virulence as seen in tests *in vitro* (8) in which the biochemical properties are also changed (11).

Both the natural resistance of staphylococci and that acquired *in vivo* may raise serious clinical and epidemiological problems. Even though germs adapt themselves to penicillin with greater difficulty than to the sulphonamides, we have to face the same problems which were raised by sulphonamide-resistant gonococci and streptococci in the pre-penicillin period, and we must entertain the possibility that the development of resistant strains or the selective survival of primarily resistant strains may come to limit the usefulness of this antibiotic.

We have been particularly interested in the occurrence of primarily penicillin-resistant staphylococci in the nose and throat of human beings. Recent and numerous investigations have plainly shown the great importance of air-borne infections, particularly cross-infections in hospital, and have thereby given present-day interest to this problems to which one of us also in earlier studies (12, 13, 14, 15) has tried to find a solution.

Material and Methods.

Strains of Staphylococci, isolated from the upper respiratory passages of persons with clinically healthy throats, and of patients suffering from angina faucium, scarlatina and diphtheria were examined. A few strains isolated from pyogenic lesions of the skin, notably impetigo, furunculosis and superficial abscesses, served as controls.

After being grown in pure culture on blood-agar dishes, the strains were examined for haemolysis, pigment-formation and capacity to coagulate plasma. *Rammelkamp's* method (16) was then employed for the determination of penicillin-sensitivity, a standard penicillin solution in 0.85 % saline solution containing 20 u/ml. being first prepared. In a series of 11 tubes, serial dilutions of 0.20 ml. of the standard solution of penicillin were made with 0.20 ml. of ordinary nutrient broth. To each tube in the series 0.5 ml. of the proper dilution of a 18-hour culture of the test organism was added. The cultures containing penicillin were then incubated at 37° for 18—24 hours, after which they were examined for turbidity. In this way penicillin-sensitivity was examined in relation to concentrations ranging from 0.01 u/ml. to 5.71 u/ml. In each case a control was established with series of the »Oxford« strain as test microbe.

Results.

In this way altogether 316 strains of staphylococci were examined, — 287 from nose and throat and, as controls, 29 strains from the pyogenic lesions of the skin already referred to.

All the strains induced haemolysis, formed pigment, and coagulated plasma. The combination of these three properties in one and the same strain is practically proof positive of a staphylococcus being pathogenic (13, 17, 18). Exceptions to this rule are very rare. Table 1 gives the penicillin-sensitivity of the strains examined.

Table 1.
The Penicillin-Sensitivity of 316 Strains of Pathogenic Staphylococci.

	Nr. of strains	Penicillin-Sensitivity		
		0.005 - 0.08 u/ml.	0.17 - 5.71 u/ml.	5.71 u/ml.
Staphylococci from nose and throat	287	208 (72.5 %)	25 (8.7 %)	54 (18.8 %)
Staphylococci from pyogenic processes	29	21 (68.5 %)	3 (10.5 %)	5 (17.2 %)
Total	316	229 (72.4 %)	28 (8.9 %)	59 (18.7 %)

We have classified the strains in three groups according to their penicillin-sensitivity. A strain is regarded as penicillin-sensitive when its growth is inhibited in concentrations up to 0.08 u/ml., and it is considered as absolutely resistant when it is insensitive to a concentration of 5.71 u/ml. Between these limits are the comparatively rare groups of strains which are inhibited in concentrations of 0.17 - 5.71 u/ml., so the transition from sensitive to resistant strains is gradual.

Our observations on staphylococci from throat and nose will be seen to tally quite well with the investigations, already referred to, of staphylococci from pyogenic lesions.

A certain number of strains of staphylococci were also isolated from patients who had just undergone treatment with intramuscular

Table 2.

The Penicillin-Sensitivity of Pathogenic Staphylococci Isolated from the Nose and Throat of Persons who had or had not been Treated with Penicillin.

	Nr. of strains	Penicillin-Sensitivity		
		0.005 - 0.08 u/ml.	0.17 - 5.71 u/ml.	5.71 u/ml.
Staphylococci isolated from untreated persons	265	202 (76.1 %)	21 (8 %)	42 (15.9 %)
Staphylococci isolated from penicillin-treated persons	22	6 (27.3 %)	4 (18.2 %)	12 (54.5 %)

injections of penicillin in doses of altogether one to several million units. Here we found, as was to be expected, a comparatively greater number of penicillin-resistant strains.

Although the number of strains in this group is small, it is noteworthy that more than half of them were penicillin-resistant. We are still investigating this problem.

In 13 cases, the staphylococci were examined before and after penicillin treatment of the same patient. In 11 cases, the results were the same before and after treatment, the strains in 10 of these cases being penicillin-sensitive (6 derived from the upper respiratory tract and 4 from pyogenic processes) and the eleventh being absolutely resistant. In 2 cases there was an increase of resistance, in the one case from sensitivity to absolute resistance, in the other from relative to absolute resistance. The possibility of a cross infection cannot be denied with certainty as the strains were not serologically typed or bacteriophage typed, but in these cases such an event is improbable. It is conceivable that the great number of resistant strains examined after penicillin treatment reflects two factors, the first and surely the most important being a process of selection of primarily resistant strains, and the other being the development of penicillin-resistance during treatment.

Discussion.

Our material contains all the transitional stages between the most common, markedly penicillin-sensitive strains of staphylococci and the primarily markedly resistant strains. Other observers (9) have found the sensitive and the resistant strains to belong to two distinct groups, whereas others again (5) have found every variation in penicillin-resistance.

Clinically speaking, it would seem that our relatively resistant strains (tolerating penicillin concentrations between 0.17 and 5.71 u/ml.) can be classed as resistant, representing the very high proportion of about 28 per cent. of our material. They were unaffected by concentrations of penicillin in the blood or tissues between 0.1 and 0.2 u/ml. To raise the concentration of penicillin would probably be of but limited value as it has been found that (5) above a certain level of 1 ± 0.8 u/ml. an increase of the concentration hardly augments its action.

It is evident that in clinical medicine more and more importance will be attached to the determination of the penicillin-sensitivity of strains of staphylococci. When a strain is found to be penicillin-resistant, penicillin may yet be given, for as in other infections (anthrax) penicillin-resistance may not prevent success with this treatment. But under such circumstances, one resorts more readily to supplementary treatment, surgical or other. And it has also been shown

that staphylococci resistant to penicillin may well be sensitive to sulphonamides (4). It has also been noted that, in the lower degrees of resistance, there is no relationship between penicillin-resistance and streptomycin-resistance even though at high levels of resistance to both we may find a slight cross resistance to these antibiotics (11).

From the epidemiological point of view we are still in doubt over the significance of the great frequency of penicillin-resistant strains of staphylococci in the throat. But it is to be feared that this state of affairs will do much to promote the frequency of primarily resistant strains. There is a genuine risk of a marked increase in the number of such strains as has occurred, for example, in the case of sulphonamide-treated gonorrhoea (19).

Summary.

1. Investigations of penicillin-sensitivity of strains of staphylococci isolated from the nose and throat have shown that there are many resistant strains. In 18.8 per cent. of 287 strains, marked resistance (over 5.71 u/ml.), and in 8.7 per cent. (between 0.17 and 5.71 u/ml.) relative resistance was found.
2. In more than half the cases examined, the strains of staphylococci isolated from the throats of patients treated with penicillin were found to be penicillin-resistant.
3. The epidemiological significance of the occurrence of penicillin-resistant staphylococci in the upper respiratory tract is not perfectly plain, but we may presume that this state of affairs will contribute much to an increase in the frequency of primarily resistant strains.

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COARCTATION OF THE THORACIC AORTA WITH AN ANEURYSM DISTAL TO THE OBSTRUCTION

By Pentti I. Halonen and Alvari Aho.

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Aneurysm of the thoracic aorta distal to the obstruction is a very rare phenomenon in the presence of coarctation of the aorta. The literature has reported, as yet, few more than ten cases, confirmed at post-mortem examination^{1, 2, 3, 4, 5, 6, 7, 8, 9, 11.}). The most frequent cause of death has been rupture of the aneurysm. There has been, in addition, at least two cases which have shown clinical evidence of such a lesion.⁹) In the following we present two cases of our own.

Case Reports.

Case 1.

A farmer, aged 20, with a healthy childhood, but who has since his 19th year suffered from headache, fatigue, vertigo, and pain in the chest. About one month prior to the fatal end, the patient caught tonsillitis and was found to have enlarged and reddened faucial glands. The blood pressure was 190/95 mm of mercury in the arms, not measurable in the lower limbs; the pulse of the femoral and popliteal arteries not palpable; the WaR and Kahn tests negative; the blood picture showed nothing of note; the SR 1 mm/1 hr; no albumine in the urine; the Nyl. test negative and nothing significant in the sediments; X-ray evidence of changes typical of coarctation: erosion of the ribs and absence of the knob of the aorta. During 3 days the patient received 9 Gms of Sulphathiazol in the form of tablets, whereupon the throat symptoms disappeared. One day later he was discharged, the SR being 10 mm/1 hr. At home the patient experienced a persistent fatigue and feeling of discomfort, which made him return to the hospital for further care after the lapse of one month. The patient's general condition was found to be exceedingly poor. A rough systolic murmur was audible in the cardiac re-

gion, the blood pressure of the upper extremities being 190/95 mm of mercury. The lungs showed no percussory or auscultatory signs of disease. The SR was now 60 mm/1 hr. X-ray examination, on admission, revealed in the left hilar region the presence of a well defined, structureless, solid mass, which appeared to be fused into the thoracic aorta. The lung tracings themselves were normal. The patient suddenly vomited a quantity of blood and succumbed.

Post-mortem examination reveals enlarged and suppurating tonsils as well as hypertrophy of the left heart. The ascending aorta is shorter than usual, the aortic arch and the vessels arising from it being normal. Distal to the left subclavian artery, at the point of attachment of the ligamentum Botalli, there is a narrowing of the aortic wall to a lumen of 8 mm in diameter. The aorta shows, further, at the site of the constriction, a narrowing ridge in the intima, 0.5 mm in breadth. The intima is intact. Close to the constriction, distal to it, there appears in the aortic wall an oval opening of 10 mm leading into an aneurysmal dilatation of considerable size (3 by 3.5 by 5 cm). The intact aortic intima extends into this sac, which is filled with a bluish grey thrombosed mass sticking to the walls of the cavity. The sac is attached to the hilus of the left lung. Its bottom shows three perforations of the size of a pin's head, through which blood has passed into the left pleural cavity, amounting to $\frac{1}{2}$ ltr at the time of the autopsy. As far as other organs are concerned, only a generalized anaemia is worth mentioning.

Histological observations: Thickening of the intima in the area of the coarctation and fibrous hyperplasia in all the layers of the aortic wall can be demonstrated. In this region, the muscle fibers of the media show an irregular course, a fan-shaped spreading into the intima. The elastic tissue does not appear to be reduced. Close to the constriction, distal to it, there is a number of microscopically detectable aneurysmas, (Fig. 1), the floors of which show fibrous hyperplasia of the intima. Completely obliterated vasa vasorum or those with thickened walls may be seen in the adventitia. A change in the histological picture is apparent in the area of the main aneurysm. The



Figure 1.

Aortic wall shows an aneurysm (a) with a few obliterated vasa vasorum (o) in its surroundings.



Figure 2.

Thrombosed mass (c) attached to walls of ruptured aneurysm, media (m) showing necrotic areas, haemorrhages, and inflammatory cell infiltration (d).

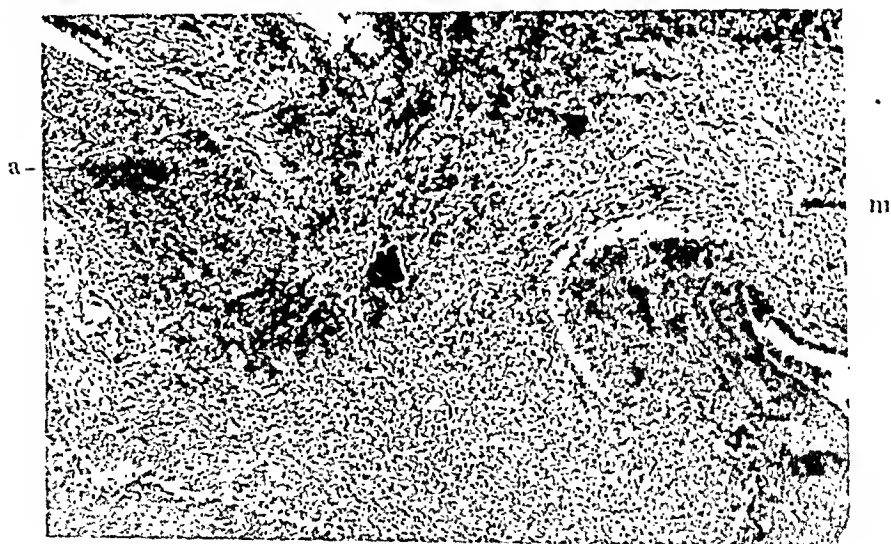


Figure 3.

Inflammatory cell reaction extending from adventitia (d) to intima. Media (m) shows necrotic areas penetrated by granulation tissue.

adventitia and the surrounding connective tissue show a marked infiltration with inflammatory cells, extending to involve also the media and intima. In the different layers of the aortic wall, there may be found scattered necrotic areas, some of which display a vegetation formed by granulation tissue. (Fig. 3). Several areas show an increase in fibrillar connective tissue and, on the other hand, a complete destruction of the elastic tissue. The inflammatory infiltration consists mainly of lymphocytes and plasma cells, but also granulocytes occur. The aneurysm itself is filled with an organized thromboid mass together with some blood, which appears in the superficial layers of the wall. (Fig. 2). The aneurysmal wall shows, further, calcified areas and deposits of hemosiderine. The stains for fat and bacteria negative. Elsewhere the aortic wall entirely normal.

Case 2.

The 39-year-old wife of a farmer. The patient had formerly enjoyed a perfect health. At the age of 37, shortly after her third delivery, she began to complain of fatigue and discomfort. Slight bleeding from the genitals made its appearance simultaneously. As no pathological signs were found in the genitals, the patient was admitted to a hospital of internal medicine. There the patient was found to have a yellowish pallor and oedematous ankles. The heart was distended to the left and an intense systolic murmur was audible throughout the heart. The blood pressure in the upper limbs was 170/95 mm of mercury. An increase in the SR, ranging from 60 to 127 mm/1 hr, was persistent. The WaR and Kahn tests negative. The blood picture revealed a severe normochromic anaemia, a mild leukocytosis, and a marked thrombocytopenia. A persisting increase in residual nitrogen. Albumin was present in the urine during the whole period of hospitalization and the sediment was that of a typical nephritis. The patient developed a suppurative otitis. The uraemia gradually grew severe and the tendency to bleed increased. The patient died, her illness terminating in the clinical picture of uraemia.

Autopsy findings: slight hemorrhages in the skin, the buccal cavity, nose and pharynx. The spleen and lymphatic glands are enlarged. The kidneys show the typical picture of nephritis. The heart is dilated, 370 Gr in weight and measuring 14 by 11 by 8 cm. The ascending aorta, the aortic arch, and the arteries arising from it are normal. A typical coarctation of the aorta is seen in the descending part of it, distal to the origin of the left subclavian artery. In the aortic wall, distal to the coarctation, in an area of 3 by 3.5 cm, there appears a number of bulbous dilatations of a pea's size. Marked ulcerations of the intima are seen in the same area.

Histological observations: The region of the coarctation is very similar to that of case 1. Oedema of the aortic wall is present here and there in the area of the aneurysm. The intima shows fibrous thickening, whereas the muscle fibres of the media have thinned. An increase in the connective tissue is apparent in the various layers of the aortic wall, but particularly in the adventitia. Fresh ulcerations are seen in the intima and slight haemorrhages in the superficial layers of the arterial wall. The adventitia, and partly also the media, is found to be the seat of perivascular inflammatory cell infiltration, which is mostly mild, but in some places wellmarked. The involved parts of the media show fresh necrotic areas as well as older, partially organized, necrotic tissue. A considerable proportion of the inflammatory cells are lymphocytes and plasmacells, but also granulocytes occur. The remainder of the aortic wall appears to be normal.

Comment.

To explain the pathogenic mechanism of an aneurysm distal to the thoracic aorta, in which blood pressure is lower than the usual, is attended with considerable difficulties. In the reported cases, the site of the aneurysm is very much the same, i. e. immediately distal to the coarctation. This leads us to search for a special factor which is responsible for the development of an aneurysm at this particular point. A reference has often been made to *Rokitansky*,¹⁰⁾ according to whom there must be a special congenital weakness of the aortic wall at this very point. *Hecker*³⁾ primarily points out the rise of the intra-abdominal pressure as the cause of a simultaneous rise in the thoracic aorta below the site of constriction and of a subsequent development of an aneurysm. In the opinion *Zaslow* and *Krasnow*¹¹⁾ the rise of the intra-abdominal pressure accounts for the rupture of the aneurysm reported above. Their theory is that a whirl-pool of the blood flow distal to the coarctation produces the dilation of the aorta and the weakening of its wall. The significance of infection is emphasized by *Reifenstein*⁹⁾ et al. The major part of the instances of aneurysm discovered by them at autopsies showed a simultaneous presence of an infectious disease and this was also the case in two instances with clinical evidence of aneurysm. The anti-infectious therapy instituted by them proved to be effective in reducing and calcifying the aneurysm.

In the cases reported by us the mechanism of the aneurysm appears to be similar. Both of them show a dilatation of the aorta distal to the coarctation, an increase in the connective tissue of the aortic wall, and destruction of the vasa vasorum. The most satisfactory explanation to this is given by *Zaslow* and *Krasnow*.¹¹⁾ A similar phenomenon, according to them, might occur in any part of the circulatory system, in case an artery should be constricted, i. e. a dilatation might develop distal to the constriction. This is pointed out by *Lenéque* and *Bruze*⁵⁾ in explaining the formation of an aneurysm distal to the coarctation. Infection may partially be responsible for the aneurysm in our cases. The first case presents signs of a persistent infection after tonsillitis. There is simultaneously X-ray evidence of an aneurysm. The histological picture reveals typical inflammatory changes in the aneurysmal wall. The second case shows the co-existence of several different infections. Histologically, there are signs of inflammation in the walls of the rising aneurysm also in this case.

Summary.

1. Aneurysm of the thoracic aorta distal to a coarctation is uncommon.

2. A case with ruptured aneurysm distal to the coarctation has been described.
3. Another case with multiple aneurysms distal to the coarctation has been described.

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ON THE FREQUENCY OF PNEUMOCOCCUS TYPES IN DENMARK 1939—1947

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In autumn 1937 the Pneumococcus Department of the State Serum Institute was established primarily for the production of antipneumococcal rabbit serum for therapeutic purposes. At the same time, serological typing was commenced of the pneumococci isolated from specimens of material sent to this department from various kinds of patients. The diagnosis was made by means of the Neufeld capsular swelling reaction. To begin with, this test was made using diagnostic sera received from the Lederle Laboratories, U. S. A. Before long, however, the diagnostic pneumococcus sera were produced in the State Serum Institute, which now for a number of years has been furnishing various European laboratories with such sera.

The occurrence of the pneumococcus types in Denmark — as evident from the material examined in the State Serum Institute was first investigated by Vammen (1940), covering the period from February 1, 1938 to July 31, 1939. Later, Mørch^{2, 3} has examined in a similar way the material received from 1939 to 1942.

The present paper gives a survey of the material examined in the Pneumococcus Department from August 1, 1939 to July 31, 1947, a total of 8 years. Until July 31, 1942, differentiation was made between the individual types within the same group (Mørch³), but afterwards the differentiation was limited to the group diagnosis.

In Denmark, since 1944, we have recognised 73 pneumococcus types (Mørch⁴), distributed as 15 type groups and 26 single types. In practice the diagnosis is made from 41 different types (or groups), numbered from 1 to 43 (Nos. 26 and 30 are not employed).

As the therapeutic sera for the pneumococcus groups are polyvalent, *i. e.*, produced by immunization with all the types within the given group, it is of no interest for the serum treatment to make the diagnosis of the individual types in the group. Likewise, the diagnostic sera for the groups are polyvalent too, reacting with all the types with-

in the group concerned. Only in cases of particular epidemiological interest is the diagnosis made on the types within the group — by means of absorbed sera, »factor sera« (Mørch¹).

The diagnosis of a given strain is facilitated by use of 8 pooled sera which react with all the pneumococcus types with the exception of Type 3, for which an unmixed serum is used, because it is difficult to produce a high titer serum of this type. Besides, Type 3 is one of the most frequent types whose diagnosis often implies certain difficulties, as cultures undergo autolysis more rapidly than cultures of other pneumococcus types.

For the period of 1939—47, in the Pneumococcus Department, we have recorded how often the individual types (groups) have been demonstrated in sputa, cultures from the throat or larynx, pus from the ear, pus from the maxillary sinus, spinal fluids, pleural exudates, blood cultures, peritoneal pus, nasal cultures, and pus from several other, different, lesions.

The total number of specimens examined for the presence of pneumococci has been about 8000—10,000 a year (Table 1). Of the yearly totals, 3000—5000 have contained pneumococci. As often more than one specimen has come from the same patient, the number of patients showing the presence of pneumococci has been somewhat lower, about 2500—3000 a year (Table 1).

Table 1.

Total Numbers of Specimens, Positive Specimens and Patients with Pneumococci examined in the Period of 1939—1947.

Year	Total No. of specimens	Positive specimens	No. of patients with pneumococci
1939—1940	9942	5009	3187
1940—1941	9005	4445	3142
1941—1942	8629	4305	2985
1942—1943	9740	4242	3118
1943—1944	10052	4229	3025
1944—1945	9313	3346	2687
1945—1946	8319	3360	2870
1946—1947	7758	2803	2454

The percentage frequency of the individual pneumococcus types per year during this period as well as their average frequency for all these 8 years are given in Table 2. In 1939—41 Type 3 was the most frequent type, while in the following years it was not demonstrated quite as frequently. Still Type 3 is one of the most frequent types. In 1941—44 Type 1 was by far the most common type, particularly in the year 1942—1943. In 1944 Type 2 became the most frequent, but Types 1, 19, 6 and 3 appeared almost just as often. In 1945—46 Type 19 was the one found most frequently, but in 1946—47 Type 3 was again

the most frequent. On calculating the averages for the 8 years we find Type 1 to have appeared most frequently, then Type 3.

Table 2.
Frequency of Pneumococcus Types 1939-1947.*)

Type	1939-40	1940-41	1941-42	1942-43	1943-44	1944-45	1945-46	1946-47	Average
1	8.8	8.6	<i>17.9</i>	<i>24.3</i>	<i>18.0</i>	10.9	6.8	8.1	<i>13.1</i>
2	3.1	3.1	3.6	6.7	8.5	<i>11.9</i>	7.8	8.1	6.0
3	<i>15.2</i>	<i>13.1</i>	11.0	8.0	10.1	10.0	10.8	<i>12.1</i>	11.8
4	2.5	2.4	3.8	2.9	2.4	2.3	3.7	3.2	2.9
5	0.7	2.1	3.9	2.4	1.8	1.7	1.3	0.7	1.8
6	9.1	12.0	10.6	9.9	8.5	10.2	10.6	9.1	9.7
7	7.4	5.4	4.0	2.9	3.0	2.9	3.2	3.7	4.5
8	4.5	3.5	2.0	2.5	2.1	2.6	2.7	3.7	3.0
9	3.1	4.3	3.2	3.1	2.7	1.8	3.0	2.1	2.8
10	2.2	2.7	1.8	2.0	1.4	1.6	2.0	2.1	1.9
11	2.1	2.2	1.8	1.9	2.3	1.7	2.7	1.7	2.0
12	0.9	0.9	1.5	1.3	1.5	1.3	0.6	1.0	1.1
13	1.8	1.3	1.0	1.4	2.0	2.1	2.3	2.2	1.7
14	2.6	4.4	4.8	4.5	3.6	5.1	4.2	4.4	3.9
15	1.8	2.4	1.5	2.3	2.6	2.5	2.8	2.4	2.3
16	1.6	0.9	0.9	0.9	0.9	1.1	1.2	1.7	1.2
17	1.2	1.7	1.1	1.7	1.5	1.2	1.5	1.7	1.4
18	3.8	3.4	3.5	3.5	4.1	4.7	4.5	4.2	3.9
19	8.9	<i>11.4</i>	8.9	7.2	10.6	10.5	<i>12.1</i>	11.5	9.9
20	1.7	0.8	0.8	0.6	0.5	0.8	1.1	1.0	1.0
21	1.3	1.5	0.6	0.6	0.5	0.4	0.7	0.9	0.8
22	2.4	1.0	1.1	0.5	0.9	2.2	2.3	2.2	1.5
23	4.5	3.7	3.5	3.0	2.8	3.2	3.6	3.5	3.3
24	0.6	0.3	0.5	0.8	0.9	1.0	0.9	0.7	0.7
25	0.4	0.2	0.2	0.1	0.2	0.1	0.03	0.1	0.2
27	0.4	0.5	0.5	0.4	0.6	0.7	0.6	0.3	0.5
28	0.3	0.1	0.2	0.2	0.3	0.4	0.6	0.5	0.3
29	1.6	0.6	0.7	0.3	0.6	0.4	0.3	0.3	0.7
31	1.1	0.9	0.4	0.5	0.6	0.5	0.4	0.7	0.7
32	0.4	0.3	0.2	0.4	0.2	0.4	0.3	0.2	0.3
33	1.2	0.7	1.2	0.8	1.2	1.1	0.9	1.0	0.9
34	1.4	1.1	1.0	0.7	0.8	0.4	1.3	1.5	1.1
35	1.0	1.5	1.2	0.7	1.2	0.9	1.6	1.5	1.2
36	0.1	0.1	0.1	0.1	0.1	—	0.03	0.2	0.1
37	0.3	0.4	0.3	0.3	0.4	1.0	0.8	1.0	0.6
38	0.1	0.3	0.5	0.4	0.2	0.1	0.2	0.2	0.3
39	.	0.2	0.03	0.1	0.1	0.04	0.1	0.04	0.1
40	.	0.03	0.03	0.1	0.1	0.3	0.3	0.2	0.2
41	.	0.03	—	0.03	0.1	0.04	0.1	0.1	0.1
42	.	0.03	0.1	—	—	—	0.03	—	0.02
43	.	.	0.03	—	—	—	—	—	0.005

*) The figures give the percentages of all patients with pneumococci in the respective years. The most frequent type in each year is italic.
 . means that the type had not yet been established.
 1939-40 = From August 1, 1939 to July 31, 1940.
 1940-41 = " " " 1940 " " " 1941 etc.

One of the reasons for this change in the relative frequency of the types from one year to another may be variation in the make-up of the material. When many sputa are examined in the course of the year, it is reasonable to expect that the pneumococcus types found in pneumonia will predominate. If the specimens come chiefly from pediatric or otologic clinics, this will influence the total result in another direction. From children with pneumonia the specimens received will generally consist of pharyngeal or laryngeal swabs instead of sputa. The pneumococcus types demonstrated in pharyngeal or laryngeal cultures come mainly from children, and thus we may expect to meet with the types found in this age-class (Types 6 and 19). In the cases of infection of the ears other types are prevailing (Types 1, 3 and 19).

Table 3.

Number of Patients with Pneumococci demonstrated in Specimens of Various Nature. 1939—1947.

Nature of specimens	1939—40	1940—41	1941—42	1942—43	1943—44	1944—45	1945—46	1946—47	Total
Sputum	915	1194	1215	1078	1613	1374	1153	852	10394
Cultures from throat or larynx	1078	1219	1096	1101	1436	1126	835	768	8659
Ear cultures	133	450	487	513	498	396	370	323	3170
Spinal fluid	35	48	39	63	54	44	68	63	414
Pleural exudate	32	40	29	63	66	59	44	34	367
Blood culture	17	20	14	23	16	9	7	4	110

From Table 3 it is evident that the distribution of the various kinds of specimens, year by year, has been fairly uniform in the period here concerned. It will be noticed that the number of pleural exudates and blood cultures with pneumococci has been decreasing in the last 3 years, whereas the number of spinal fluids with pneumococci still keeps high. Presumably the explanation of this is to be found in the circumstance that empyema and bacteriemia most often make their appearance in connection with serious cases of pneumonia. In the later years the treatment of pneumonia has been so effective that only very few pneumonic patients have serious complications. On the other hand, from the incidence of pneumococcus meningitis, it appears as if the treatment of the most frequent lesion primary to the development of meningitis — *i. e.*, otitis — has not hitherto been so successful. Possibly penicillin therapy in ear will bring about a decrease in the incidence of pneumococcus meningitis.

Table 4 shows the number of the individual pneumococcus types demonstrated in the various kinds of specimens examined in the course of 1938—1947. From each patient only one positive specimen is included in this account, so that the figures here recorded also give the number of patients under the respective headings.

Table 4.

Pneumococcus Types in Sputa, Cultures from the Throat or Larynx, Spinal Fluid, Pleural Exudates, Blood Cultures and Ear Cultures 1939-1947.')

Type	Sputa	Cultures from throat or larynx	Spinal fluids	Pleural exudates	Blood cultures	Ear cultures
1	1415	838	77	163	45	582
2	1218	322	27	27	14	75
3	1432	480	51	23	10	562
4	420	207	23	5	4	50
5	141	55	14	7	—	202
6	500	1505	43	24	7	249
7	616	275	17	18	2	66
8	442	170	8	11	2	56
9	323	273	10	10	2	70
10	219	174	6	6	1	31
11	264	175	4	1	2	20
12	163	70	11	3	4	21
13	245	139	—	3	—	13
14	133	590	20	7	2	214
15	197	288	3	2	1	35
16	156	78	4	2	—	20
17	166	138	2	5	—	16
18	324	406	31	10	3	150
19	470	1253	22	19	2	490
20	138	61	2	—	—	6
21	46	111	—	—	—	16
22	176	128	5	2	—	18
23	195	398	10	3	4	109
24	95	63	4	4	1	13
25	31	5	3	—	—	2
27	63	24	4	1	1	19
28	45	23	—	—	—	6
29	91	35	—	—	—	9
31	76	49	1	—	—	6
32	44	19	1	—	—	2
33	115	88	5	5	—	11
34	139	63	2	2	1	8
35	151	87	—	3	1	13
36	11	11	—	—	—	2
37	81	15	—	—	—	2
38	16	28	1	—	—	6
39	11	3	—	—	—	—
40	21	3	1	1	—	—
41	2	8	1	—	—	—
42	2	1	1	—	1	—
43	1	—	—	—	—	—
Total:	10394	8659	414	367	110	3170

*) The figures give the number of patients.
The most frequent type of each material is italic.

The sputa show most often Types 3, 1 and 2; then come Types 7, 6, 19 and 8. In cultures from the throat or larynx — which, as mentioned, in most cases originate from children — Types 6 and 19 are by far the predominant ones.

In the spinal fluids Types 1 and 3 are the most frequent; then come Types 6, 18, 2, 4, and 19. In the specimens of pleural exudate Type 1 is by far the predominant type, while Types 2, 6, 3, 19 and 7 are fairly frequent. Practically all the pneumococcus types have been demonstrable in the spinal and pleural fluids. In the blood Type 1 is the most frequent.

Cultures from the ear have most often yielded growth of Types 1, 3 and 19, besides rather often Types 6, 14 and 5. Type 5 is found relatively often in ear lesions. In the total material for 1939—1947 (Table 2) Type 5 is found in 1.8 %, while in the same period it amounts to 6.4 % of the pneumococci found in ear cultures (Table 3).

Table 5.
Types of Pneumococci demonstrated in Pus from Maxillary Sinus.
1941—1947.

Type	No. of patients	Type	No. of patients	Type	No. of patients	Type	No. of patients
1	8	12	2	23	17	36	—
2	1	13	8	24	4	37	1
3	19	14	9	25	—	38	2
4	2	15	4	27	—	39	—
5	1	16	5	28	1	40	2
6	24	17	9	29	2	41	—
7	4	18	8	31	1	42	—
8	7	19	19	32	2	43	—
9	8	20	5	33	9	Total: 232	
10	9	21	2	34	9		
11	9	22	12	35	7		

Most frequent types: 6, 3, 19, 23, 22.

Since 1941 many specimens of pus from the maxillary sinus have been received for examination for pneumococci — altogether from 232 patients. In this kind of specimen Types 6, 3, 19 and 23 were the most frequent, while Type 1 was relatively rare (Table 5). Thus it appears as if the type distribution is a little different in ear lesions.

In the 8-year period here concerned we received nasal cultures only from 80 patients. The most frequent types encountered here were: Types 6 (18 patients), 19 (8 patients), 23 (8 patients), 3 (7 patients). Thus we have very nearly the same type distribution for the nasal cultures as for the cultures from the maxillary sinus as probably also was to be expected.

In peritoneal cultures from 1939—1947 growth of pneumococci was found in 29 patients, all with Type 1.

Pneumococci have been demonstrated in pus from several cases of arthritis. Cultures from the hip-joint yielded growth of pneumococci of Types 2, 10 and 23, from the knee-joint in 5 patients (Types 1, 1, 10, 19, 19); and from the shoulder-joint Types 9 and 14 were isolated.

In the case of one patient suffering from osteomyelitis pneumococcus Type 1 was found in the pus. In 5 cases pneumococci were demonstrated in pus from the frontal sinus, namely: Types 1, 13, 14, 23 and 33. In a case of orbital infection, pneumococci of Type 35 were obtained. From the conjunctiva Types 3, 6, 14 and 15 have been grown. Type 10 was found in a dental cyst, Type 3 in an abscess of the brain.

In a patient suffering from endocarditis pneumococcus Type 25 was demonstrated. Vaginal cultures from two patients showed pneumococci of Type 1 (presumably patients suffering from pneumococcal peritonitis). Finally, pneumococcus Type 19 was found in pus from the rectum in one case, Type 20 in pus from abscess of the foot in another case.

In several cases more than one pneumococcus type was demonstrated in the same patient. Thus in 1942—1947 more than one type was found in 552 patients, namely:

519 patients with 2 types,				
30	»	»	3	»
3	»	»	4	»

In the years prior to 1942 the occurrence of more than one type in the same patient was relatively more frequent than later on, but in many cases this phenomenon involved the finding of some of the new types that reacted with more than one of the diagnostic sera employed. Thus, for instance, Type 11 A reacted both with serum 11 and serum 16.

In the Pneumococcus Department, in 1943—1947, we received 112 specimens of urine (from 109 patients) for examination for pneumococcal carbohydrate. Only in 3 patients was type-specific carbohydrate of Type 1 demonstrated, and only in 1 patient carbohydrate of Type 2. The majority of these specimens came from patients with peritonitis. Roesgaard (1945) has shown that type-specific precipitation of pneumococcal carbohydrate in the urine is positive only if the pneumonic process is fairly extensive. Otherwise the excretory aspects are variable so that positive and negative reactions in the same patient may alternate daily. Thus a negative reaction does not warrant the inference that the patient has no extensive pneumococcus infection. In the case of one patient, in which we had not been able to demonstrate the presence of pneumococcal carbohydrate in the urine, pneumococcus Type 1 was found on the following day in pus from the peritoneal cavity. As the technique of the test for pneumococcal carbohydrate is rather complicated, and as the method has yielded only 4 positive reactions in the course of 4 years, we have now given up this form of examination as a routine method.

The Pneumococcus Department also receives blood samples to be examined for their pneumococcus antibody content. In 1943—47 we received altogether 402 such specimens of blood from 354 patients. Only 28 of them showed the presence of pneumococcus antibody. The small number of positive blood samples is most likely attributable to the circumstance that the blood samples chiefly originate from patients who on preceding examination of the sputa had not shown the presence of pneumococci. In no instance has pneumococcus antibody been demonstrated in the blood of a patient in whose sputum no pneumococci had been demonstrable suggesting that the method employed for examination of the sputa for the presence of pneumococci is quite sufficient.

The serum from the blood sample is examined both for capsular swelling and for agglutinating antibodies. The Neufeld test for capsular swelling is carried out with 9 mixtures of vaccine, reacting together with all pneumococcus types. When capsular swelling is observed in one of these mixtures, the examination is continued with the individual types in the mixture. When the type has been determined, the capsular swelling and agglutinating titers are estimated. Most often the titers are 4—32. The presence of pneumococcus antibody has been demonstrated for the following types:

<i>Type</i>	<i>No. of patients</i>
1	6
2	11
3	1
4	2
7	2
8	1
15	1
18	1
19	1
20	1
23	1
	<hr/>
Total	28 patients

Sera from several patients have given nonspecific capsular swelling with pneumococcus Types 27 and 28. In most of these cases the serum has given a positive reaction with both types simultaneously. Usually the reaction has been rather weak, with a capsular swelling titer of 2—8. It is characteristic of this nonspecific reaction that in the same sera no agglutination reaction is seen corresponding to a distinct capsular swelling. Löfström (1943) states that the nonspecific reaction disappears as soon as specific antibody is formed. Here, however, it would be appropriate to mention the case of a patient with pneumococcus pneumonia Type 2, whose blood simultaneously contained specific antibody for Type 2 and also showed nonspecific cap-

sular swelling with Type 28. On 3 successive days this patient showed the following capsular-swelling and agglutinating titers:

		Capsular- swelling titer	Aggluti- nating titer		Capsular- swelling titer	Aggluti- nating titer
April 13	Pn. 2	16	8	Pn. 28	8	0
" 14	"	8	8	"	8	0
" 15	"	4	2	"	4	0

As will be evident from the number of specimens examined (Table 1) in Denmark considerable interest is still being taken in the type determination of pneumococci. Largely, the specimens here received are of the same character from one year to another, the greater majority consisting of sputa and cultures from the throat and ears (Table 3). The Pneumococcus Department aims to keep on diagnosing pneumococci and typing the strains within the 41 types (or groups) mentioned, just as the production of diagnostic pneumococcus sera will be continued.

Summary.

An account is given of the results of type determination of pneumococci within 41 types or groups during 8 years in the Pneumococcus Department of the State Serum Institute.

The frequency of the types is subject to variation from one year to another. On an average, however, the most frequent types are: 1, 3, 19 and 6. The composition of the material varies but slightly from one year to another. In the past three years the occurrence of pneumococci in pleural exudates and blood cultures has been decreasing, whereas the number of patients with pneumococci in their spinal fluid has not decreased — in spite of the superior therapeutic methods.

The interest taken in the typing of the pneumococci found in pus from the ears still keeps fairly great; here the most frequent types are: 1, 3 and 19. In pus from the maxillary sinns, Types 6, 3 and 19 are found most frequently. The same type distribution applies to the nasal cultures.

On examination of specimens of urine for their pneumococcal carbohydrate content, a positive reaction has been found but in very few cases.

Examination of the blood for the presence of pneumococcus antibody is only of slight practical significance, as a positive reaction is found only in patients with demonstrable pneumococci in their sputum or other pathological material.

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ACTION OF MITOTIC POISONS IN VITRO I EFFECT OF URETHANE ON DIVISION OF FIBROBLASTS

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Introduction.

In the nineteen twenties A. P. Dustin² discovered that some substances had a particular nucleotoxic effect on certain cells apparently without damaging the cytoplasm. Such compounds Dustin called »poisons caryoclasiques«. Especially through the works on colchicine by Dustin³ and Ludford⁴ it has been shown that it is dividing cells that are destroyed, and accordingly such substances are now generally termed *mitotic poisons*. These compounds have become topical through a large number of papers on the clinical action of various substances with inhibitory effect on neoplastic disorders. It is the object of this and subsequent articles to report on some data from this field.

Urethane was introduced in treatment of leukemia by E. Paterson *et al.*¹² in 1946. Although it has now been proved that urethane treatment in most cases is inferior to roentgen therapy, the way of action of this compound is still of considerable theoretical interest.

The chemical constitution of urethane is $\text{NH}_2\text{COOC}_2\text{H}_5$ (ethyl carbamate). As the first Warburg¹⁵ found in 1910 that *phenyl urethane* (ethylphenyl carbamate) inhibits at metaphase the mitoses of eggs from sea urchins (*Strongylocentrotus lividus*). A similar effect on plant cells was demonstrated by Lefèvre⁸, Simonet & Guinoche¹³, and corroborated by Templeman & Sexton¹⁴ who also found that *isopropylphenyl carbamate* could inhibit the growth of grain germs, and that this substance is three times as potent as phenyl urethane. On the other hand, urethane has no growth-inhibiting effect on plants.

Haddow & Sexton⁷ found that both phenyl urethane, isopropyl urethane, and urethane inhibit the growth of Walker's rat carcinoma, and that among these urethane has by far the most pronounced effect.

So far few investigations have been carried out into the way of action of urethane, and a discrepancy of opinion exists as to the results.

Haddow & Sexton⁷ found that urethane causes a transient increase

in the number of mitoses in the Lieberkühn crypts of the mouse, which in their opinion may be explained as a retardation of the mitotic cycle at all phases.

P. Dustin^{4, 5} found that after intraperitoneal injection of urethane in doses of 1 g/kg body weight the number of mitoses in the Lieberkühn crypts of the mouse was reduced by two thirds. The effect began ten hours after injection and continued for 48 hours and differential counts of mitoses showed a normal distribution of the various phases. Simultaneously with the reduction in mitoses *Dustin* observed a large number of cells with pyknotic nuclei. In *Dustin's* opinion these pyknotic figures were cells damaged just before mitosis. He supported this view by stating that the increase of pyknotic cells roughly corresponded to the decrease in the number of mitoses.

By examination of patients suffering from myeloid leukemia *Moeschlin*¹¹ found that following urethane treatment the number of mitoses in the bone marrow and the spleen was reduced by 50 per cent. The fall was evenly distributed over the various phases of mitosis.

*Ludford*⁹ investigated the effect of urethane on tissue cultures of mouse carcinoma and renal tissue from mouse embryos. If one drop of a 2 % urethane solution was added to the culture, an arrest of mitosis at metaphase occurred in some of them. However, it was not possible to obtain uniform results, and in many of the cultures no effect at all could be demonstrated.

*Geiersbach*⁶ investigated the effect of urethane on cultures of heart fibroblasts from chick embryos. A comparatively high urethane concentration was necessary to produce any effect. In concentrations of 1—3 % no normal mitoses occurred. A number of large, round, dark-stained cells were present, in which the nucleus had been replaced by a clump of chromatin, and these cells were taken for mitoses which had been arrested and subsequently destroyed. Degenerative changes of resting cells such as vacuolization of the cytoplasm and rounding-up of cells were found in the urethane concentrations mentioned.

Thus all investigators agree that urethane has an inhibitory effect on mitosis, but opinions differ as to the mechanism of this inhibition. According to *Haddow & Sexton*⁷ urethane causes a retardation of the mitotic cycle at all phases, but arrest does not occur at any particular phase. Contrarily *Ludford*⁹ found that the mitoses were arrested at metaphase. *Geiersbach*⁶, too, believed that an arrest occurred at some phase or other, but he did not deal with the problem in detail. Both *Dustin*^{4, 5} and *Moeschlin*¹¹ found that urethane inhibits the mitotic activity so that fewer cells undergo mitosis, but they did not find an arrest at any particular phase.

The object of this paper is to further clarify the way of action of urethane on cell division by means of differential countings of mitotic phases in tissue cultures subjected to varying concentrations of urethane during varying periods of time.

Material and Methods.

Fibroblasts from eight-day chick embryo hearts were used for the experiments presented here. This tissue is well suited for studies on mitosis as the cultures always contain an abundance of mitoses. In addition, it is comparatively easy to obtain a uniform growth in the various cultures, which gives a good material for comparison. The nutrient medium consisted of one drop of adult chicken plasma and one drop of chick embryo extract. Urethane solutions were prepared from a stock solution of 20 % in saline being diluted with Tyrode's solution. The tissue was grown as coverslip cultures according to the usual technique.

Urethane Added to Growing Cultures. When after 48 hours the growing zones of the cultures had become sufficiently large, five drops of urethane were added, thus producing the concentration desired in the cultures. The procedure was as follows: The coverslip was removed, one drop of urethane solution was added to the culture and the additional four drops were placed in the concavity of the slide so that it was almost filled. The coverslip was replaced and sealed with paraffin. The controls were treated correspondingly with five drops of Tyrode's solution, and all cultures were replaced in the incubator. At intervals a number of the cultures were fixed in Carnoy's fluid and stained with hematoxylin. The number of mitoses per 1000 fibroblasts was counted. The figures in the following tables are average values for several cultures (mostly six).

Urethane Added at the Explantation. In some cases urethane has been added to the nutrient medium already at the first explantation in order to observe the effect on the growth of the tissue. For such experiments cultures were prepared in one drop of adult chicken plasma, one drop of chick embryo extract, and one drop of urethane solution. In these experiments there was no difficulty in making the plasma clot in spite of the fact that it had been diluted by the addition of the urethane solution.

Effect of Different Urethane Concentrations on the Number of Mitoses.

A. Detailed Comments on Tables:

The object of the experiments reported was to examine the effect of different urethane concentrations on the number of mitoses. Urethane was added to two days old cultures.

Urethane 0.35 %.

The total number of mitoses corresponded to that of the controls. The differential count of the various mitotic phases did not present any abnormalities.

Table 1.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 0.35 %				Control			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
2 hours	31	7	17	7	51	9	17	13
4 hours	31	4	17	10	41	9	17	15
6 hours	43	7	22	14	28	5	13	10
8 hours	33	5	18	10	38	7	19	12
24 hours	17	3	7	7	25	4	13	8

Interpretation. In this concentration urethane had no effect on mitosis.

Urethane 0.66 %.

The *total number of mitoses* largely corresponded to that of the controls.

On the other hand, by *differential counting* of mitotic phases slight changes were found. After 4 hours the number of *prophases* was slightly reduced, and it remained low during the rest of the experimental period, lasting 24 hours in all. During the first 8 hours there was a slight increase of the number of *metaphases*, but after 24 hours it corresponded to the controls. After 6 hours the number of *ana- and telophases* was slightly reduced.

Table 2.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 0.66 %				Control			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
2 hours	43	4	26	13	28	3	15	10
4 hours	33	1	22	10	37	7	18	12
6 hours	38	2	28	8	33	5	17	11
8 hours	38	3	26	9	41	7	18	16
24 hours	24	1	18	5	22	5	10	7

Interpretation. The reduction of the number of prophase indicates that fewer cells than normal entered mitosis. That the total number of mitoses was not reduced is explained by the fact that the reduction of prophase was »compensated« by the increased number of metaphases. The increased number of metaphases suggests that there was a slight inhibition of mitosis at this phase. The reduced number of ana- and telophases also suggests that the mitoses proceeded beyond metaphase only with difficulty. However, as the reduction of the number of ana- and telophases was but slight, there is every reason for believing that after being inhibited for some time at metaphase the mitotic process can nevertheless be completed. It must then be assumed that the time in which the cells were at metaphase was somewhat longer than normal.

The changes in the differential counts were yet so slight that they cannot be taken as significant. But since they showed the same tendency as those which plainly appeared in higher urethane concentrations, the above interpretation of the results seems justifiable.

Urethane 0.8 %.

In the *total number of mitoses* there was an increase which began 4 hours after the addition of urethane. It reached maximum after 6 hours, and then the number of mitoses decreased. After 13 hours it was again almost normal, and after 24 hours it was considerably lower than normal.

In order to find out the reason for these fluctuations of the number of mitoses, it is necessary to examine the *differential count* of the mitotic phases. Already 2 hours after addition of urethane there was an essential fall in the number of *prophases*. During the entire experimental period the number of prophase was 0—1. The majority of all mitotic cells (approximately 90 %) was at *metaphase*. This increased percentage of metaphases was brought about, partly by a fall in the number of cells at the other phases, and partly by an absolute increase of the number of metaphases. The increase occurred already 2 hours after addition of urethane, became more pronounced in the course of the following hours, and reached maximum after 6 hours. After that time the number of metaphases decreased, and after 24 hours only a few metaphases were found. The number of *ana- and telophases* was during the first 6 hours normal or slightly reduced. After that time a marked reduction of the number of cells at this stage occurred.

Interpretation. The disappearance of prophase indicates that the mitotic activity was greatly reduced. The even increase in the number of metaphases signifies that there was an arrest at this stage, and

that the cells accumulated at this phase. The fall of the number of metaphases 13 hours after addition of urethane must be explained by the inhibitory effect of urethane on the mitotic activity, which now became predominant. The explanation of the gradual disappearance of *ana- and telophases* must be that mitosis could not proceed beyond metaphase.

Table 3.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 0.8%				Control			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
2 hours	44	1	30	13	33	5	17	11
4 hours	50	0	43	7	39	6	17	16
6 hours	87	1	76	10	34	8	18	8
13 hours	45	0	40	1	45	8	19	18
24 hours	8	0	5	1	27	6	13	8

Urethane 1 %.

In the *total number of mitoses* an increase occurred, which began 4 hours after urethane had been added. The increase reached maximum after 6 hours; after that time the number decreased again. After 10 hours the number of mitoses was again almost normal, and after 24 hours it was lower than normal.

The *differential count* showed essentially the same conditions as were found in a urethane concentration of 0.8 %. The number of prophases had fallen after 2 hours and remained low throughout the experiment. During the entire experimental period there was a greatly increased percentage of *metaphases*, brought about partly by a fall in the number of cells at the other phases, partly by an absolute increase of the number of metaphases. Just like in a concentration of 0.8 % the increase of the number of metaphases commenced after 2 hours and reached maximum after 6 hours. It reached a somewhat higher level than in a concentration of 0.8 %. Subsequent differential counts showed a decrease of metaphases. During the first 4 hours the number of *ana- and telophases* was slightly reduced, after 6 hours markedly so. The reduction was more pronounced than in a concentration of 0.8 %.

Table 4.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 1 %				Control			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
2 hours	37	2	26	9	33	7	16	10
4 hours	62	0	57	5	33	5	15	13
5 hours	92	2	87	3	29	5	15	9
10 hours	33	0	32	1	34	7	17	10
24 hours	17	0	16	1	28	4	16	8

Interpretation. The disappearance of pro-phases suggests a lowered mitotic activity. The increase of the number of metaphases indicates an arrest at this phase. The fact that the fall in *ana- and telophases* was more pronounced in a concentration of 1 % than in 0.8 % suggests that the inhibition at metaphase was stronger in the higher concentration.

Urethane 1.2 %.

The total number of mitoses was very low after 2 hours, then it increased, and reached maximum after 6 hours.

The differential count showed that *pro-phases* rapidly disappeared. Nearly all the mitoses were at *metaphase*. Two hours after addition of urethane the total number of metaphases was somewhat lower than normal; after that time the number of metaphases increased to over normal values, but did not reach so high a level as in a lower concentrations. After 6 hours *ana- and telophases* had completely disappeared.

Table 5.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 1.2 %				Control			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
2 hours	8	0	8	0	38	8	18	12
4 hours	43	1	41	1	31	6	16	9
6 hours	63	0	63	0	27	6	13	8
12 hours	49	0	49	0	32	7	15	10
24 hours	24	1	23	0	25	5	13	7

Interpretation. The low number of mitoses 2 hours after addition of urethane suggests a reduced mitotic activity. The disappearance of prophases also suggests that fewer cells than normal entered mitosis. That the number of cells that accumulated at metaphase owing to the arrest at this stage was somewhat lower than in concentrations of 0.8 % and 1 % may indicate a lower mitotic activity in a urethane concentration of 1.2 % than in lower concentrations. The complete disappearance of ana- and telophases indicates a total mitotic stop at metaphase.

Urethane 1.5 %.

During the first hours the *total number of mitoses* was at the upper limit of the normal, then it rapidly fell to nil.

The *differential count* showed an accentuation of the changes observed in the lower urethane concentrations. The *prophases* had disappeared already 15 minutes after addition of urethane. Simultaneously the number of *metaphases* had become twice as large as normal, and remained so, for 4 hours, after which time the metaphases disappeared. From now onwards there were no mitoses at all. The number of *ana- and telophases* had all the time been nil in most cultures.

Table 6.

Time after addition of urethane	Number of mitoses per thousand cells							
	Urethane 1.5 %				Control			
	Total count	Pro- phases	Meta- phases	Ana- and telo- phases	Total count	Pro- phases	Meta- phases	Ana- and telo- phases
15 min.	36	0	36	0	33	7	14	12
30 min.	31	0	31	0				
60 min.	52	0	52	0	48	8	21	19
2 hours	35	0	34	1	33	5	17	11
4 hours	38	0	38	0	35	5	17	13
6 hours	0	0	0	0	35	6	18	11

Interpretation. The rapid disappearance of prophases indicates a complete inhibition of the mitotic activity in this urethane concentration. The increased number of metaphases was due to the fact that the cells which had entered mitosis when urethane was added accumulated at this phase. As no new mitoses occurred, the constant number of metaphases must indicate that nor did any mitoses proceed to ana- and telophases. The arrest at metaphase must then have been

complete. This assumption is also supported by the fact that the number of ana- and telophases was very low, in most cases nil.

B. Survey.

The range of mitotic inhibition of urethane is rather narrow, i. e., between 0.66 % and 1.5 %.

In concentrations lower than 0.66 % the cultures behave exactly as the controls. In concentrations of 1.5 % or more all the cells will

URETHANE 1%

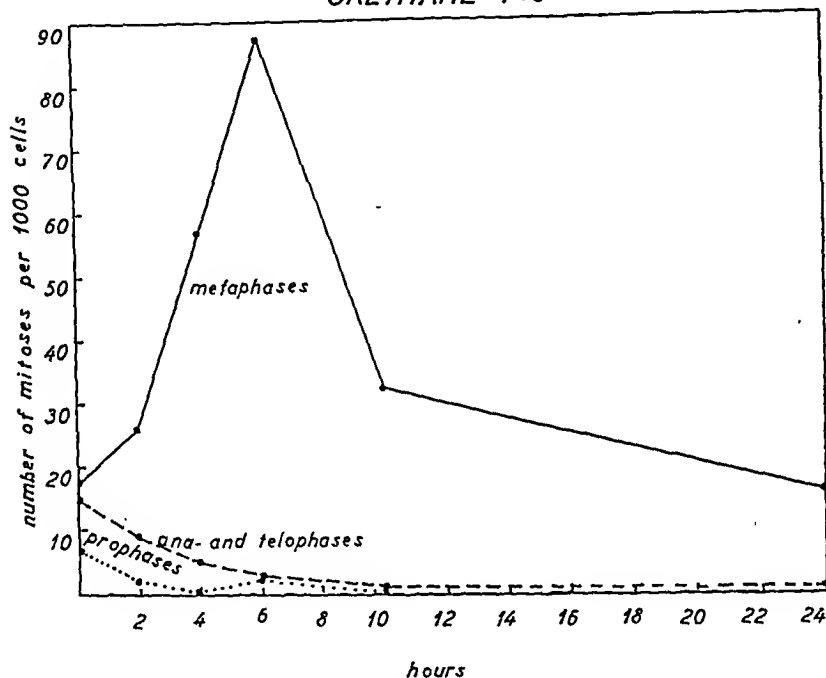


Fig. 1.

Effect of urethane on mitosis at various times.

Ordinate: Number of mitoses per 1000 cells. Abscissa: Number of hours after addition of urethane. — The inhibition at metaphase (increased number of metaphases and reduced number of ana- and telophases) becomes more intense with the time in which the cultures have been exposed to the action of urethane. — The inhibition of mitotic activity (reduced number of prophases) soon becomes apparent. Mitotic activity falls further during the experiment.

soon be damaged to such an extent that only during the first hours it will be possible to speak of a special mitotic effect.

Urethane affects mitosis in two ways: 1) *Mitotic activity is reduced*, i. e., fewer cells than normal commence mitosis. 2) *Mitosis is inhibited at metaphase*.

In the differential count the low mitotic activity manifests itself in a reduction of the number of prophases. The inhibition at metaphase becomes evident by an accumulation of cells at this stage with

a simultaneous fall in the number of ana- and telophases. The number of cells that accumulates at metaphase as a consequence of the arrest at this stage is, on the one hand, dependent on how intense the mitotic inhibition is, on the other hand, on how much the mitotic activity has been reduced.

The diagram (Fig. 1) shows the effect of urethane on mitosis at various times as found in the differential counting of mitotic phases. The inhibition at metaphase (increased number of metaphases and reduced number of ana- and telophases) becomes more intense with the

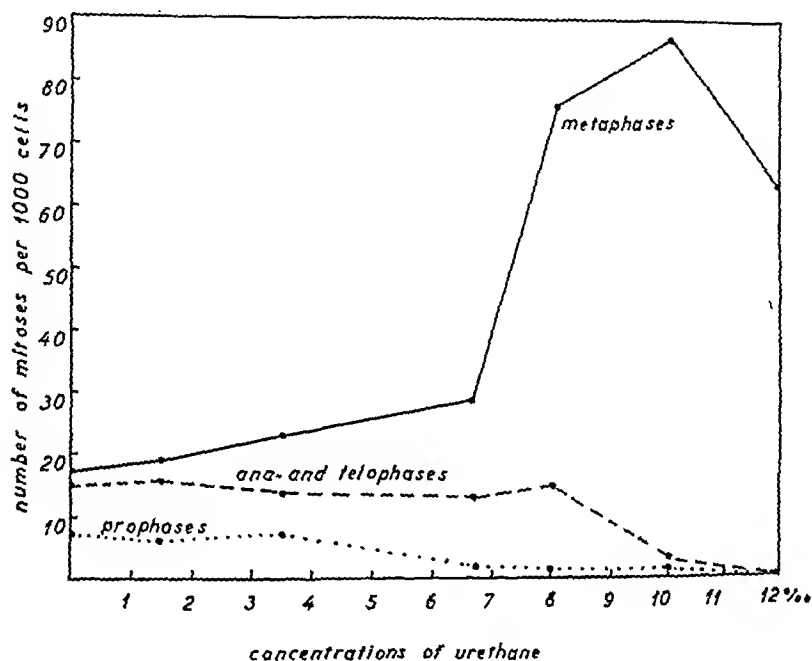


Fig. 2.

Effect of different urethane concentrations on mitosis.

Ordinate: Number of mitoses per 1000 cells 6 hour after addition of urethane. Abscissa: Concentrations of urethane. The inhibition at metaphase (increased number of metaphases and reduced number of ana- and telophases) and the inhibition of mitotic activity (reduced number of prophases) intensify with increasing strength of the urethane concentrations.

time in which the cultures have been exposed to the action of urethane. The inhibition of the mitotic activity (reduced number of prophases) becomes apparent soon after addition of urethane. When the curve representing the number of metaphases shows declining values 10 hours after addition of urethane, i. e., at a time where the arrest at metaphase according to the low ana- and telophase values should be almost complete, this must indicate that the mitotic activity at that time is lower than during the first part of the experiment.

The diagram (Fig. 2) shows the effect of different urethane con-

centrations on mitosis as found in the differential counting of mitotic phases. — *The inhibition at metaphase* (increased number of metaphases and reduced number of ana- and telophases) becomes more intense with increasing urethane concentrations. In a concentration of 1.2 % the number of ana- and telophases is nil, i. e., the arrest at metaphase is complete in this concentration. *The inhibition of the mitotic activity* (fall of number of phosphases) becomes more intense with increasing urethane concentrations. When the curve representing the number of metaphases shows declining values in concentrations of 1.2 % or more, i. e., in concentrations where the arrest at metaphase according to the ana- and telophase values should be complete, this must also mean that the mitotic activity has been reduced to a greater extent in high concentrations. In a concentration of 1.5 % the inhibition of the mitotic activity is complete.

It is thus seen that the intensity of the two effects which urethane exercises on dividing cells: reduction of the mitotic activity and arrest at metaphase, is proportional to the strength of the urethane concentration and the time in which the cells have been exposed to the action of urethane.

Cytological Action.

Mitoses.

In concentrations of 0.35 % or less no effect of urethane was seen.

In the lowest active concentration, 0.66 %, there was an inconsiderable reduction of the mitotic activity and a slight inhibition at metaphase; the mitoses were morphologically normal.

In the higher concentrations, 0.8—1.5 %, considerable morphological changes at metaphase were seen. The changes found may be interpreted as a progressive degeneration at metaphase. The types of metaphases found may be divided into 5 groups:

1) Normal metaphases. In the early part of metaphase the chromosomes were scattered throughout the cytoplasm (Fig. 3). In the last part of metaphase the chromosomes formed a normal equatorial plate, in which individual chromosomes could be distinguished, and the spindle was discernible (Fig. 3).

2) In some of the cells the chromosomes of the equatorial plate had fused, forming a compact disk. In some places of this disk a few individual chromosomes could still be distinguished. In addition, some of the fibres of the spindle could be seen (Fig. 4).

The remaining types of metaphases found in the cultures were abnormal and more or less degenerated.

3) A common type of abnormality was failure of some of the chromosomes to become attached to the equatorial plate (Fig. 5). The chromosomes remaining outside the equatorial plate tended to contract and bunch together (Figs. 6—8). In such cells fibres of the



Fig. 3.



Fig. 4.



Fig. 5.

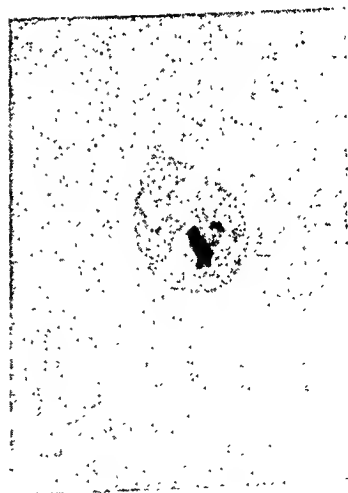


Fig. 6.

Figs. 3—10.

Progressive pyknosis in mitoses arrested at metaphase.

Fig. 3: Normal metaphases. Fig. 4: Metaphase with formed equatorial plate with incipient coalescing of chromosomes. Fig. 5: Abnormal metaphase: some

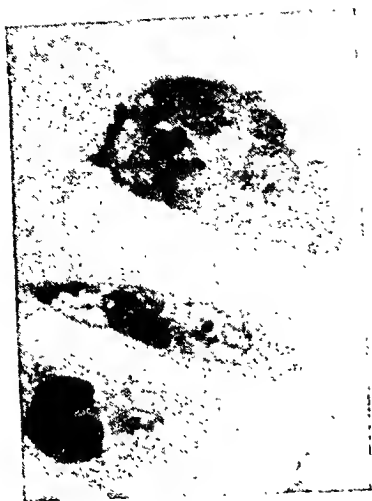


Fig. 7.



Fig. 8.



Fig. 9.



Fig. 10.

chromosomes have remained outside the equatorial plate. Figs. 6—8: Abnormal metaphases with chromosomes remaining outside the equatorial plate and with coalescing of chromosomes. Figs. 9—10: Markedly pyknotic metaphases.

spindle could still be seen, but in the photographs it proved impossible to identify the spindle.

4) Gradually as the chromosomes fused the equatorial plate was reduced to a chromatin bunch in the middle of the cell. The cytoplasm was dark as in pyknotic cells (Fig. 9). In the dark cytoplasm it was still possible to see remnants of the spindle. Thus it cannot be doubted that cells of this appearance really were metaphases.

5) Later on the metaphases assumed a still more pyknotic appearance. The chromosome bunch had decreased in size, the cytoplasm was very dark staining, and the spindle had disappeared (Fig. 10).

The conditions in urethane concentrations between 0.8 and 1.2 % were essentially uniform, and accordingly the morphological changes in these concentrations will be described collectively: Two hours after addition of urethane a fairly equal distribution was found among a) metaphases with normal equatorial plate (Fig. 3), b) metaphases with formed equatorial plate with incipient pyknosis (Fig. 4), and c) metaphases with abnormal equatorial plate with incipient pyknosis (Fig. 5). After 4 hours most frequently abnormal metaphases with more or less advanced pyknosis were found as is seen in Figs. 5, 6, and 7. After 6 hours the markedly pyknotic types as shown in Figs. 8 and 9 were in excess. After 24 hours only types as shown in Figs. 9 and 10 were found.

In a urethane concentration of 1.5 % the pyknotic changes occurred much earlier. After 15 minutes nearly exclusively abnormal metaphases as in Figs. 6—9 were found. After 2 hours all metaphases were markedly pyknotic as in Fig. 10.

Interpretation. The changes described may be explained as a progressive pyknosis in mitoses which have been arrested at metaphase. The mitotic process *can* proceed to the end of the metaphase with the formation of a completely normal equatorial plate, but it is, however, possible that this occurs only during the first couple of hours after addition of urethane. Most frequently abnormalities in the formation of the equatorial plate occur, as some chromosomes remain outside the equatorial plate.

In all these cells a mitotic spindle has been formed. According to *Ludford's* investigations on sodium cacodylate and colchicine the arrest at metaphase produced by these compounds was due primarily to the failure of the mitotic spindle to form and function in the normal manner. The studies presented here show that an arrest at metaphase may occur even though a mitotic spindle has been formed.

When the cell has been held up at metaphase for some time, it degenerates. The chromosomes coalesce as the pyknosis progresses. This applies both to the chromosomes in the equatorial plate and those remaining outside the equatorial plate. At the same time the cytoplasm stains deeply. Not until the mitoses have reached a certain degree of pyknosis does the spindle disappear.

There may be two causes of the degeneration of mitoses arrested at metaphase. It is possible that a cell which has been impeded in its normal function is unable to survive. Furthermore, urethane may have a direct toxic effect on the metaphase, so that in addition to the hold up at this stage it also causes death of the cell. That at least the latter condition is present can be seen from the fact that a strong urethane concentration, 1.5 %, induces pyknosis much earlier than the lower concentrations. This does not, of course, exclude the possibility that both factors may be operative as the cause of pyknosis in the lower concentrations.

Non-dividing Cells.

Urethane concentrations which do not affect mitosis, 0.35 % and less, also leaves non-dividing cells intact.

The lowest urethane concentration which affects the mitoses, 0.66 %, does not seem to influence the morphology of the resting cells.

The urethane concentrations which had a strong injurious effect on mitosis, 0.8—1.5 %, also affected to some extent non-dividing cells, but not at all to the same degree as the mitoses.

When the cultures had been exposed to the action of urethane in concentrations of 0.8—1.2 % for 6—10 hours, slight damage to a few of the resting cells initiated. In these cells vacuoles were present in the cytoplasm. Some of the cells became pyknotic: They were small and round, and had lost contact with the other fibroblasts; the small, deeply stained nucleus was excentrically lodged, but had preserved its nuclear structure. These changes were especially seen in the peripheral parts of the cultures. After 24 hours a considerably larger number of pyknotic cells and cells with vacuoles in the cytoplasm were found. At that time about one half of the fibroblasts showed these degenerative changes. The rest appeared quite normal.

In a concentration of 1.5 % the degenerative changes initiated in a few cells already after 15 minutes. The number of degenerated cells increased gradually, and after 6 hours the majority of the fibroblasts were pyknotic.

Interpretation. In concentrations of 0.8—1.2 % urethane had a markedly injurious effect on the mitoses. The morphology of resting cells was also affected by these urethane concentrations, although to a less degree. When resting cells had been exposed to the action of urethane for 24 hours, degenerative changes occurred in some of them, but the majority were, however, morphologically normal.

Effect of Urethane on Cell Emigration.

The growth of a culture is dependent on two factors: 1) The emigration of cells from the explant. 2) The multiplication of emigrating cells by mitosis. A substance possessing a selective destructive action

on the mitoses should accordingly allow a certain growth of the cultures, though with a smaller zone of outgrowth than normal, since that part of the growth of the cultures which is due to emigration has been preserved. If the substance also inhibits that part of the function of the cell which induces emigration, no growth occurs.

As urethane exercises an injurious effect on the mitoses, while a great many of the non-dividing cells do not undergo morphological changes, the following experiments were performed in order to examine the emigratory power of the cells.

Cultures were grown in a urethane-containing medium, and their zones of outgrowth were compared with those of the controls.

Urethane Concentration without Mitotic Effect. In cultures with a urethane concentration of 0.2 % no inhibition of growth was seen after 48 hours.

Urethane Concentration with Weak Mitotic Effect. In cultures with a urethane concentration of 0.66 % growth was slightly reduced after 24 hours, and considerably reduced after 48 hours (approximately 50 % of the growth of the cultures).

Urethane Concentration with Pronounced Mitotic Effect. In cultures with a urethane concentration of 1 % there was no growth at all after 48 hours.

Interpretation. The failure of cultures with a urethane concentration of 1 % to produce any growth at all indicates that besides damaging the mitoses urethane also damaged resting cells so much that they became incapable of emigrating from the explant, even though many cells remained morphologically normal.

It is impossible to say whether the reduced growth in a urethane concentration of 0.66 % was due exclusively to the mitotic inhibition, or whether it was also, to some extent, due to a reduction of the emigratory power of the fibroblasts.

Is the Damaged Function of Non-dividing Cells Irreparable?

It cannot be expected that urethane should affect the cells of the organism in the same constant way as it does in the cultures. The cells of the organism will be under the influence of urethane only for a certain length of time, which is, at least in part, determined by the proportion between the absorption and elimination of urethane. It is therefore important to demonstrate whether the damaged function of non-dividing cells is irreparable. In order to investigate this problem the following experiments were made.

Urethane was added to some growing cultures as in the experiments already described: The cultures were allowed to grow in a concentration of 1 %, and were then transferred to a normal urethane-free medium. After another 48 hours the zones of outgrowth of these cultures were compared with those of the controls.

It appeared that cultures which for 24 hours had been exposed to the action of urethane were capable of continuing their growth in a urethane-free medium, and that after the next 48 hours the growth corresponded to that of the controls.

Interpretation. Even after 24 hours' exposure to the action of urethane in concentrations capable of inhibiting mitosis the fibroblasts were able to continue their normal growth when the urethane was removed from the cultures. Accordingly the action of urethane had not inflicted permanent damage on the non-dividing cells.

Stability of Urethane in Cultures.

In order to be able to evaluate the results of the experiments it is important to know whether the urethane concentration remains constant throughout the experiments. The following experiments were made to elucidate this. It has been attempted to employ the mitotic effect as an indicator of the urethane content of the nutrient medium. At a time when urethane had exercised a definite influence on mitosis, the supernatant fluid was added to other cultures, which did not contain urethane:

To some cultures a urethane solution was added, so that the concentration in the culture became 1.2 %. After 6 hours the effect on mitosis was as described above for this urethane concentration. The fluid from these cultures was now added to urethane-free cultures, four drops to each culture. Thus the urethane concentration should be 0.8 %, provided that the original amount of urethane was still present in the fluid. After 6 hours the same effect on mitosis was seen as in the cultures where the urethane concentration was 0.8 % after addition of fresh urethane solution (Table 7).

Table 7.

Time after addition of urethane	Number of mitoses per thousand cells							
	Solution from old cultures. Presumed urethane content: 0.8 %				Fresh solution. Known urethane content: 0.8 %			
	Total count	Pro-phases	Meta-phases	Ana- and telo-phases	Total count	Pro-phases	Meta-phases	Ana- and telo-phases
6 hours	75	0	74	1	87	1	76	10

Interpretation. These experiments show that even at a time when urethane had exercised a definite influence on mitosis, it remained in the cultures, its concentration not being appreciably reduced.

Comments.

The present investigations on the influence of urethane on fibroblasts *in vitro* have shown that urethane has a general cytotoxic effect. However, in certain concentrations, 0.66—1.2 %, urethane inflicts

damage mainly on dividing cells. It inhibits the mitotic activity and arrests the cells at metaphase.

In the said concentrations a great many of the resting cells will appear morphologically normal. Certain functions of the resting cells must nevertheless, in spite of the normal appearance of the cells, be damaged. This appears from the fact that the fibroblasts were unable to emigrate from the explant when the cultures at the explantation contained urethane in a concentration capable of inhibiting mitosis. The damage to resting cells is, however, not irreparable, not even after 24 hours, since the cells can continue their growth in a normal manner when the urethane is removed from the cultures.

Urethane must therefore be classified as a mitotic poison.

In *Dustin's* opinion mitotic poisons may be divided into 2 groups:

1) Substances preventing the onset of mitosis (trypaflavine effect or radio-mimetic effect). 2) Substances arresting mitosis at metaphase (colchicine effect). Urethane, however, possesses both these effects

Urethane differs from most other mitotic poisons in two ways: 1) Rather high concentrations are required to obtain mitotic inhibition 2) The margin between the concentration inducing mitotic inhibition and that inducing pronounced cytotoxic damage is fairly narrow.

As mentioned in the introduction *P. Dustin*⁴ found in *in vivo* experiments on mice that the mitoses disappeared almost completely from the Lieberkühn crypts 10 hours after injection of urethane. In analogy with these experiments the investigations presented here show that urethane *in vitro* induces a pronounced reduction of the mitotic activity. Simultaneously with the disappearance of mitoses *Dustin* found, however, a large number of pyknotic cells, which, in his opinion were cells which had been destroyed at a stage just prior to prophase. In our tissue cultures a large number of pyknotic cells were found after 10 hours' action of urethane. These pyknotic cells were, in all probability, cells that had degenerated at metaphase. A gradual transition was found in the cultures from early metaphases and abnormal equatorial formations to these pyknotic cells. Firstly, the individual cultures contained transitional types of these cells, secondly, it was observed that the number of pyknotic mitoses and completely pyknotic cells increased with the time in which the cultures were exposed to the action of urethane. These findings support the view that the markedly pyknotic cells found in the cultures were pyknotic metaphases.

It will be noticed that the urethane concentration required to induce inhibition of mitosis in cultures was fairly high, namely 0.66—1 % which by far exceeds the concentrations that can be obtained in the organism during a urethane treatment. It may be explained by the varying degree of susceptibility to urethane found in different tissues. and bone marrow is presumably, particularly if leukemically hyperplastic, more susceptible than fibroblasts.

According to *Moeschlin's*¹¹ investigations different cells vary in susceptibility to urethane. He demonstrated that in the normal organism cells of the myeloid series did not respond to the usual urethane doses, 4 g daily. On the other hand, the number of lymphocytes decreased. Leukemic cells in patients with myeloid leukemia responded to urethane treatment with a decrease so heavy that *Moeschlin* stated that urethane had a selective effect on myeloid leukemic cells.

But even though the susceptibility of leukemic tissue and fibroblasts to urethane may differ, the mechanism of mitotic inhibition may nevertheless be identical. These *in vitro* experiments with fibroblasts to urethane may differ, the mechanism of mitotic inhibition principles of the effect of urethane on cell division.

Summary.

1) Investigations on the action of urethane on cell division have been carried out by means of differential countings of mitotic phases in tissue cultures of chicken embryo fibroblasts subjected to varying concentrations of urethane during varying periods of time.

2) Urethane has a general cytotoxic effect. In certain concentrations, 0.66—1.2 %, urethane inflicts damage mainly on dividing cells. Mitosis is affected in two ways: 1) Mitotic activity is reduced, i. e., fewer cells than normal enter mitosis. 2) Mitosis is arrested at metaphase. The intensity of these effects is proportional to the strength of the urethane concentration and the time during which the cells have been exposed to the action of urethane.

3) Mitosis is arrested late in metaphase at the time of formation of the aquatorial plate. The chromosomes are contracted. It is a characteristic feature that some chromosomes remain outside the equatorial plate. A spindle is formed, but nevertheless the chromosomes cannot separate and form anaphase. The chromosomes coalesce forming a dense chromatin bunch in the middle of the cell.

4) Many non-dividing cells show degenerative changes, such as vacuoles in the cytoplasm and rounding-up of cells, but the majority of the them remains morphologically normal.

5) In spite of the normal appearance certain functions in the non-dividing cells must nevertheless be damaged. This appears from the fact that the cells are unable to emigrate from the explant when the cultures at the explantation contain urethane in a concentration strong enough to inhibit mitosis.

6) The damage inflicted on the non-dividing cells is not irreparable, the cells being capable of continuing their growth in a normal manner when the urethane is removed from the cultures.

7) The urethane content remains constant in the cultures during the experiments.

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ON TISSUE INDUCTION

By *Gustav Levander*.

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One of the most important tasks when attempting to explain the reactions during a healing process is to find out the causal factors. The question then immediately arises as to whether the newly formed tissue emanates from the original tissue only or whether some other originally non-specific material might possibly also play a part. An answer to this question is obviously of no little importance therapeutically. If new tissue is formed from the original tissue, then it is necessary, if we wish actively to speed up the healing process, to stimulate the old cell groups to a more intense proliferation. We can for example use some kind of non-specific stimuli which produces only a general growth of tissue, as for example embryonic extracts, autolytic products, chemicals, etc. But if the new tissue regenerates from an originally non-specific blastema — for example an omnipotent mesenchymal tissue — then it will be necessary somehow to add the component specific of that tissue. These questions can of course be answered only experimentally.

Experimental methods.

At an ordinary histological examination of a healing wound it is very difficult to settle the origin of the newly formed cells. This is true both of a simple injury to the skin and, for example, a bone fracture or an injury to a muscle. The primary and the newly formed tissues are so intimately connected that we can never decide with certainty the exact dividing line and relationship between old and new. It is necessary, therefore, in order to study the different reactions during regeneration, to try other methods. The most suitable course of action in in-vivo experiments is transplantation. This method, however, has not always been used with the necessary discrimination. Many investigators simply grafted on the tissue — the regenerative power of which was to be examined — and then generally after a rather long period of observation examined the preparations. If regeneration was found to be present they concluded straight away that the new tissue was a direct proliferation of the transplanted cells. To draw such an important conclusion merely

from the premises and results of such an experiment is somewhat rash. To get at the very mechanism of growth itself it is necessary to examine closely the different stages of reaction which occur in and around the implantation. A priori it should be far from certain that a transplanted material can continue to live in the new medium. Merely the fact that we wholly detach the tissue from its sources of nutrition, represents a serious intervention in the vitality of the tissue. Under such circumstance we cannot simply presume that the vitality of the tissues *increases* on transplantation and that the cells start proliferating by dividing. The most we can expect is that a tissue isolated from its bloodvessels can retain its vitality for a certain time. The autolytic ferments soon begin their destructive activity. Neither can we expect an implanted tissue to achieve immediate nutritive contact in its new surroundings. On the contrary, it faces during the first stages a certain resistance to continued growth in the form of various histiocytic and exudative reactions, which occur on the site of implantation in order to remove all physiologically foreign tissue. Only at a later stage can we see a vascular connective tissue, growing into the graft. It is evident, therefore, that we must observe very carefully the mutual reactions which take place between the graft and its surrounding area during the earliest stages after a transplantation. Such an exact morphological analysis demands that the grafted tissue may be easily distinguishable from the newly formed tissue in the site of implantation. It is therefore preferable to use only one relatively great lump of tissue. We should avoid the mistake of using crushed tissue or, as some have done, emulsions of tissue. With such preparations there is of course no possibility of discriminating transplanted and newly formed tissue as both are mixed. A single piece of tissue, however, allows us, at least during the first stages, to follow precisely the changes through which the graft passes, and at the same time to observe the changes which take place in the surrounding tissue.

It would certainly be useful in solving this problem if we could somehow mark the grafts during implantation experiments in order to identify them more easily and isolate them from the different cell reactions which arise in the new milieu. It would also be of great importance if we could prepare the lumps of tissue in such a way that their state of vitality could be judged before the implantation. With this in view experiments have been made with bone killed by boiling. These experiments, however, have never resulted in any regeneration of bone. This is also the case with bone fixed in alcohol or formalin. The fact that *Nageotte* and *Polletini* obtained bone regeneration with fixed bone was due to their special technique. They placed their fixed bone grafts into the external ear of a rabbit. Under such circumstances we cannot exclude the possibility that the bone formation emanates from the cartilage of the ear. If, however, we implant fixed bone subcutaneously in any other part of the body we do not obtain any regeneration (*Polletini, Levander*). A method has recently been published which is a refinement of that used for these implantation experiments. *Grefberg* has shown that muscle grafts can be prepared with trypan-blue in such a way that a regeneration can, nevertheless, occur. If a piece of muscle is placed in a solution of trypan-blue the external layers are coloured blue diffusely. This shows that the superficial muscle fibres of the graft are in all probability already dead at the moment of transplantation. By staining the preparations by *Domagk's* method we can afterwards obtain a clear difference in colour between the grafted lumps of muscle and the regenerated muscle which appears later around the graft.

I shall now give a short description of some implantation experiments performed in accordance with these principles. The description is supplemented by some extract experiments on bone tissue. I shall confine myself to only two kinds of tissue which are related to each other both physiologically and embryonically, namely bone and musculature.

BONE TISSUE

There are at present really only two schools of thought, namely the periost theory and the so-called metaplastic theory. The former theory states that in the soft parts — periost, bone marrow and the content of the Haversian canals — surrounding the hard bone there are always young undeveloped bone cells (osteoblasts), which can, when necessary, develop bone-forming activity. This means from a casual point of view that the specific bone-forming energy necessary for the regeneration of bone tissue is deposited in these young osteoblasts. They are if we use an embryogenic term stable determined. Any kind of stimulation, even a non-specific one, causes an osteoblast to develop into bone tissue. The metaplastic theory, on the other hand, rules out any such pre-existing osteoblasts and emphasizes instead that bone can develop out of non-specific connective tissue. The supporters of the metaplastic theory could not, however, explain the reason why the bone forms from connective tissue. Recently, however, *Levander* has bridged this causal gap in the metaplastic theory by proving that a substance extractable by alcohol from bone tissue and bone marrow can activate young connective tissue into forming cartilage and bone.

The periost and the mesenchymal zone of growth.

On account of the varying structure of the periost at different ages, I have for several reasons considered it desirable to distinguish even in name between the periost in the young and in adults. The internal layer, rich in cells of the periost — normally termed the cambium layer — acts as a zone of growth and must, therefore, like the other growth zone — the epiphysis — be considered as actual bone tissue. The histological structure of this cambium layer resembles that of ordinary mesenchymal tissue. This tissue rich in cells is bounded outwardly by a membrane consisting of cell-deficient connective tissue. In full grown adults the zone of growth disappears, and the membrane of connective tissue remains. Only this membrane, which during the period of growth separates the growth zone, and later the definitive bone tissue, from the surrounding soft parts, ought to be termed the periost. Consequently the periost has the same function as a capsule surrounding a parenchymatous organ. The mesenchymal growth zone must, on the contrary, be considered as part of the actual bone tissue.

Now, we know from very many experiments that transplanting the periost of adults does not result in any new bone formation. *Baetzner* carried out such a series of experiments on 56 dogs with consistently negative results. The periost has, therefore, no osteogenetic power. Because of its proximity to the bone tissue, it plays a certain rôle in the extension of the callous tissue in as much as it can limit the spread of the formation of new bone.

The outcome of transplanting the mesenchymal zone of growth in the young is quite different. Ever since the experiments of *Heine* and *Ollier*, we know from considerable experience that bone regeneration occurs after both free and stalked grafts from this layer. These experiments are very important as they are to a great extent responsible for the idea of specific osteoblasts. As transplantation of this growth zone, rich in cells, resulted in bone regeneration, it was simply assumed axiomatically that the formation of bone was a result of a continued growth of the transplanted cells in the new medium. As a rule the period of observation was very long — from weeks to months — and no accurate analysis of the tissues was made. If, however, the preparations are examined carefully at a very early stage after the transplantation, the results are quite different.

Implantation experiments.

In previous papers the present writer has described experiments with implantation of the different skeletal parts into a soft medium — musculature or subcutis. By implanting the mesenchymal growth

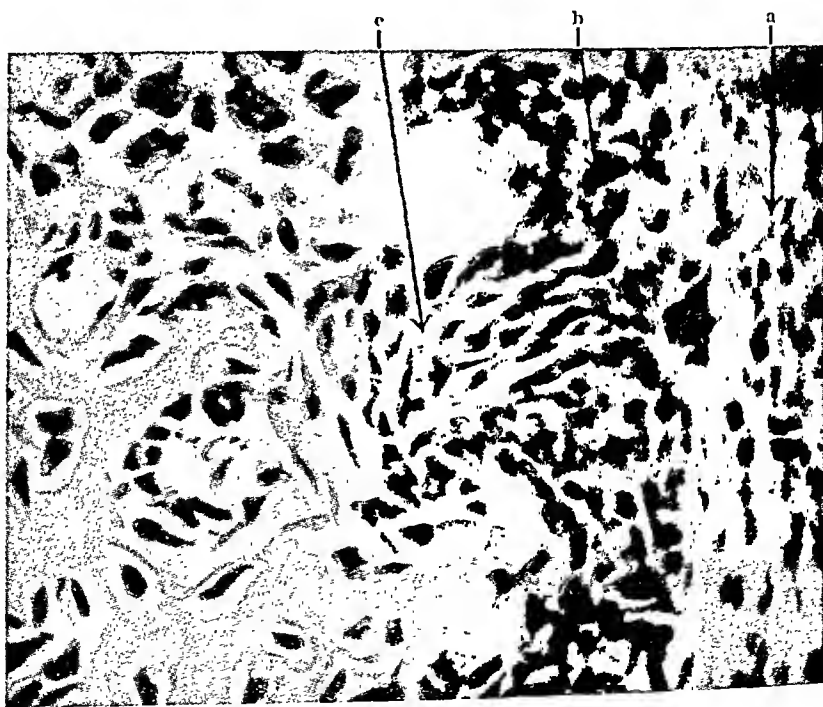


Fig. 4.

Bone in process of formation after implantation of the mesenchymal growth zone. Period of observation 5 days. a) mesenchymal tissue newly formed on the site of transplantation. b) zone of growth. c) young bone in process of growth.

zone the microscopic examination of the implantation preparations reveals that degenerative changes soon occurs in the graft — only two days after the implantation the structure of the nuclei become indistinct. After five days the first signs of a bone regeneration appears. This bone formation always starts peripherally in the young tissue, newly formed on the site of implantation, and has no connection with the grafted tissue. In fig. 1 we see how the cells from the newly formed non-specific tissue migrate towards an elongate zone, consisting of densely packed nuclei surrounded by only a scanty amount of protoplasm. From this zone of growth the osteogenesis starts.

Similar experiments with the hard bone tissue without its periosteum shows in point of principle the same results. The grafts die a few days after implantation and bone formation appears around the graft on the sixth day. There are no signs that the bone-regeneration takes place from the orifices of the haversian canals at the surface of the graft. In contrary, we sometimes find young bone growing at some distance from the graft outside in the surrounding tissue.

By implanting bone-marrow we find after four days all the nuclei of the graft transformed into irregular scattered pyknotic lumps. After six days the first signs of osteogenesis appears. They occur on the border between the graft and the surrounding tissue. It is evident — see fig. 2 — that the young bone fragments emanate from the tissue,

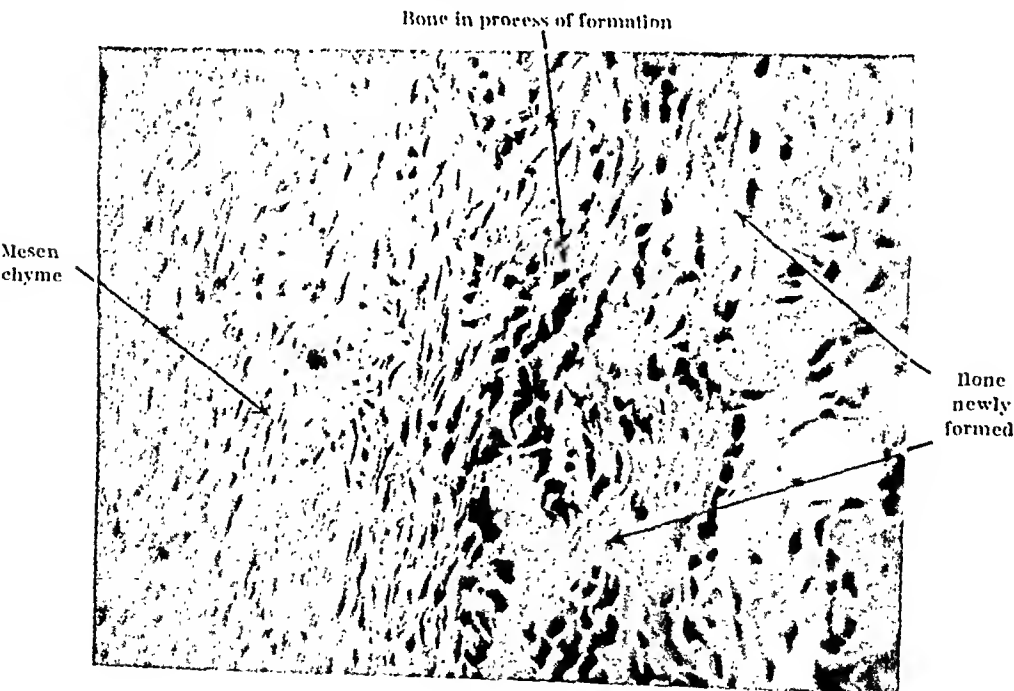


Fig. 2.
Bone-formation in the mesenchyme after grafting of Bone-Marrow. 6 days after implantation.

rich in vessel and nuclei, newly formed on the site of transplantation. It is of interest to notice the embryonic appearance of the newly formed bone with the large nuclei surrounded by an inner brighter zone — endoplasma — and an outer darker field — exoplasma — containing the stabilizing products. (See also fig 1).

Experiments with extract of bone tissue and bone marrow.

Notwithstanding the fact that careful morphological analysis of tissue reaction on transplantation of different skeletal parts shows, that bone cells in the graft do not continue to grow in the new medium and form the new bone, there is, nevertheless, every reason to believe that some connection exists between the graft and the regenerated bone. I thought, therefore, of the possibility that the dying bone graft might emit a substance capable of activating the young mesenchymal tissue in the surrounding areas into forming new bone. In order to prove the existence of this hypothetical agent, I prepared an extract of bone and injected it into a medium of soft tissue.¹⁾

In the first series of experiments aqueous extracts were used. The results were negative. I then decided to resort to alcoholic extracts on the assumption that the substance might possibly belong to the lipid group. Out of 70 experiments on rabbits, using extracts either of pure alcohol or alcohol acidified with hydrochloric acid, I obtained formation of cartilage or bone on the site of the intramuscular injections in 22 % of the cases. Simultaneous experiments with injection of pure alcohol or alcohol extracts from connective tissue or musculature into 80 controls did not result in a single case of bone regeneration. The first to verify these experiments was *Annersten* (1940). He used mainly acidified alcoholic extract, which he prepared in various ways. Out of 108 attempts to test the activity of the extracts, *Annersten* obtained formation of bone or cartilage in 45 cases. It is of interest that he was able to prove that the active factor is soluble in benzene and oil. He also carried out a number of controls. For these he used both pure alcohol and alcoholic extract of liver and muscle, and in some cases even added calcium and phosphorus. In 4 out of 102 controls formation of cartilage or bone was obtained.

Bertelsen thought it of interest to see how extracts from the various skeletal layers. react. He therefore carried out several different series of experiments. Besides preparing extract of all the layers of the bone tissue, he isolated bone marrow, corticalis, epiphysis and periost and made separate extracts of each of these strata. As solvent he used acidified alcohol. All the experiments were homoplastic. The best result was obtained with the marrow. In 12 different cases cartilage or bone

¹⁾ I shall not deal with these experiments in detail here, but merely give some of the most important results.

formation appeared 10 times — 83 %. With extract of corticalis only, positive results were obtained in 5 cases out of 12. Epiphyseal extract gave 4 positive cases out of 12, as did also periost from young animals. With extract of all skeletal parts, cartilage or bone formation was obtained in half the cases — 12 experiments. *Bertelsen* thus obtained cartilage or bone formation in altogether 29 cases out of 60 = 48.3 % \pm 6.4.

In his control experiments *Bertelsen* used both acidified and pure alcohol, and alcohol extracts of muscle, liver, suprarenal body, brain and bile. Osteogenesis was obtained once in 37 experiments. He also tried ergosterol and Vitamin D four times with negative results. In all, osteogenesis occurred in one case — liver extract — out of 41 experiments.

Lacroix describes cartilage and bone formation after homoplastic injections of extract in rabbits. He prepared an alcoholic extract from the epiphyseal cartilage of new born rabbits, and could then show »the presence of all the structures which may be analysed in a growing long bone« on the site of injection.

I am at present engaged — in collaboration with *Willstaedt* — in trying out heteroplastic extracts of bone marrow. We use bone marrow from calf and inject the extract into rabbits using my original method. We divided the lipid substance in the bone marrow by saponification into (a) the unsaponifiable fraction, (b) the fatty acid fraction, and (c) the residual solution after saponification and extraction of (a) and (b). Fraction (c) was totally inactive, but the other two fractions gave slight positive results. As the results of these experiments were not on the whole encouraging, we returned to the original method of extraction with acidified alcohol. The alcoholic extracts are then fractionated by different methods and various dissolutions in benzene, ether, petroleum-ether, etc. We have obtained active extracts and positive results in 45 to 75 % of the cases in several series of such experiments.

THE STRIATED MUSCULATURE

Unlike those regarding the regeneration of bone, the prevailing theories on the regrowth of striated musculature are fairly uniform. The regenerated parts are thought to emanate from the primary musculature. It is only on the method by which the new musculature is actually formed that opinions differ.

It is generally held that a regeneration of muscle is always based on muscle degeneration, both in purely toxic and in traumatic injuries. The state of affairs has given rise to a theory which is linked up with the so-called sarcolytic conception. During the degeneration of a muscle only the contractile elements are destroyed, while the nuclei and some sarcoplasm preserve their vitality. The parts of the

muscle thus surviving are transformed into so-called »Muskelkörperchen«, which absorb the degenerated contractile substance by phagocytosis, and become sarcolytes. The sarcolytes occur within the old sarcolemmal sheath. The central parts of the sarcolytes degenerate, while the peripheral parts are transformed into sarcoblasts and form the new muscle fibres. This form of muscle generation has also been called the *discontinuous* type.

As opposed to the sarcolytic idea, we have the theory of regeneration through so-called bulbous ends. The new muscle elements are thought to grow out of the uninjured part of the muscle fibre in the form of fibres or »plasmodia«. In contrast to the preceding theory, this kind of muscle regeneration has been termed *continuous*. The theory of direct proliferation from the original, living musculature in both toxical and traumatic injuries prevails more and more over that of *discontinuous* regeneration.

When investigating the tissue reactions during muscle regeneration, the relatively small areas comprising the actual cut surface in traumatic injury and the immediate neighbourhood of the muscle fibres in toxic damage have normally been used. Within these small areas a large number of different reactions evidently accumulate. *Frantz, Stout and Clarke* write »as a matter of fact, in the inflammatory reaction which follows the injury it is difficult to be sure of the changes in the muscle in the presence of the cellular infiltration«. *Pfuhl* also emphasizes the same facts and points out that just because of them it is necessary to search for new ways of solving this difficult problem.

Further, it is worth noting that we seldom find a clear presentation of the problem itself in the writings of those who have devoted themselves to its study. They left it wholly to the microscope to settle the problem, and often used the greatest possible magnification. The difficulty of interpreting the observations accurately has already been stressed. For this reason, our theories must of course be restricted in value for the time being.

As far as we can judge there is no reason to assume that different tissues and organs regenerate by means of different mechanisms. A comparison with other tissues ought, therefore, to be of value when framing the problems concerning the regeneration of muscles. In addition, we should try to find a fundamental parallel between embryonic development and postfoetal regrowth. Even here there is no reason for us to believe that two different mechanisms exist. It seems reasonable, therefore, to compare the regeneration of musculature with that of bone tissue. From the standpoint of embryology, both have developed from mesenchymal rudiments.

We have seen how the newly formed bone tissue emanates from a mesenchymal medium. We therefore ask ourselves in what way is mesenchymal tissue connected with the regeneration of musculature?

The problem of muscle regeneration is then reduced to the following: can musculature regenerate from a mesenchymal blastema or does it grow out of the pre-existing muscle tissue? When faced with such a question, we must of course select for our investigation a material able to give a satisfactory answer. The experiments must be so arranged that we have both a rich mesenchymal medium and degenerated musculature. The muscle preparations we obtain after injection of alcohol extracts of bone, provide just such suitable material for the study of these questions.

Injection experiments.

Intramuscular injections of alcohol in a concentration of 30—40 % produce more or less widespread muscle damage and, in addition, abundant formation of young mesenchymal tissue rich in vessels. The injections were made into the rectus femoris in rabbits. Fine regeneration pictures are obtained if a 10 % solution of bile is added to the alcohol. Too high concentrations of bile result in too widespread necrosis. It is most effective to make two injections of 2 cc alcohol-bile with an interval of two days. The preparations were taken eight days after the second injection. They give varying pictures. Both the degenerative and the regenerative changes vary considerably in extent

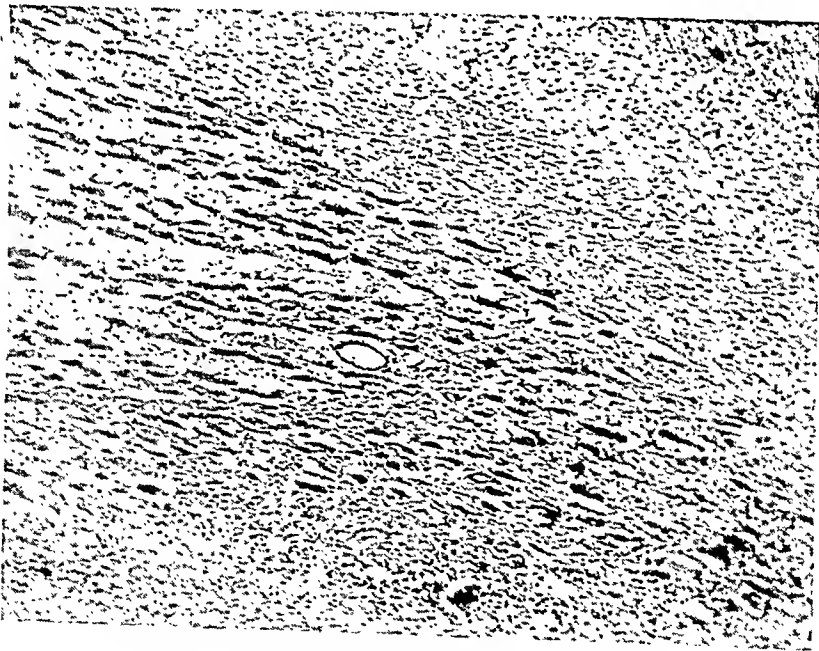


Fig. 3.

Newly formed muscle spindles in a mesenchymal medium and arranged like a »shoal of fish«.

and relative position. It often happens that the newly formed musculature lies like small uniform spindles close to the injured or living preformed musculature. On account of this more or less intimate contact it is, of course, impossible to judge the relationship between the pre-existing musculature and the newly formed muscle tissue. On the other hand, it is not difficult to find pictures in which we can clearly distinguish between old and new muscle tissue. It is not uncommon to find the regenerated elements arising like small spindles grouped like a shoal of fishes. A more detailed morphologic analysis of such »muscle shoals« may be of interest. Fig. 3 gives a general survey. Not infrequently these formations are triangular in shape. We find small spindle-like muscle fibres roughly uniform in size and shape and at fairly constant distances from one another. Even a rapid survey shows that it is a question of systematically organized tissue and not an irregularly growing histiocytic granulation tissue. We meet with the same picture everywhere, even in serial sections. No contact exists with the preformed tissue, and the muscle spindles do not touch one another. This fact appears still more clearly with somewhat greater magnification — see fig. 4. The spindles lie fairly far apart, each surrounded by a mesenchymal medium. It is of interest to observe that the ends of the muscle fibres are, so to speak, fringed into the surrounding medium. There is, therefore, no marked boundry between muscle and mesenchymal tissue, so that the transition is diffuse and the individual muscle fibres fade indiscernably into the fine meshes

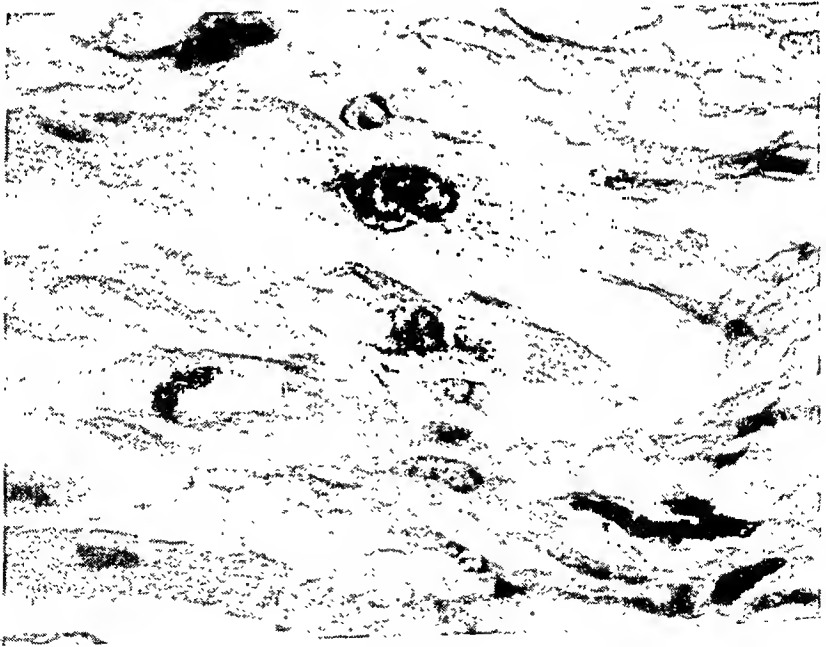


Fig. 4.

The same prep. as fig. 6 in greater magnification.

of the mesenchymal net. Alongside the muscle spindles, however, the boundry with the surrounding tissue is more sharply marked. Further, we observe the large nuclei with clearly defined chromatinic net so typical of young muscle fibres.

Discussion. We have a triangular field of young muscle spindles of equal size. The lateral delimitation of the field consists of connective tissue only. By using serial sections we can observe that the limits in the planes which are invisible in the picture also consist of connective tissue. The basis extends into the preformed musculature, which is for the most part necrotic as a result of the action of alcohol. A direct morphologic contact between the young muscle spindles and the preformed musculature cannot be observed. I have convinced myself of this by a careful examination of serial sections. From a theoretical point of view it is possible to imagine that the muscle spindles have arisen — in connection with the injections — as a result of part of the original muscle sets having become scattered and torn loose from their connections and afterwards taking up positions outside in the newly formed mesenchymal tissue. If this were the case, we ought of course to have observed fully-developed musculature and the new growth side by side. But we find only young muscle tissue. The large nuclei also prove that we have here very early stages of formation. Further, we see that all the young spindles within the field of vision are of about the same size, and must for this reason be considered to be in the same phase of development. If the young fibres had emanated from some special matrix consisting of preformed musculature, we should have expected to find muscle fibres in various stages of development, with the youngest rudiments closest to the presumable growth zone, and the more developed tissue further away from it. The even distribution of young muscle spindles of equal size within the entire field of vision argues unquestionably against such a growth mechanism.

Therefore, the morphological analysis of the tissue reaction in these injection experiments gives the following results. The earliest muscle rudiments are completely surrounded by a mesenchymal blastema. We can clearly see how the young individual muscle fibres branch out into the finely-meshed mesenchymal net. On the other hand, we find no contact anywhere between the young regenerated muscle and the primary musculature. For these reasons a mesenchymal genesis seems more probable than a proliferation from preformed muscle tissue.

Implantation experiments.

In order to study further the question of muscle regeneration I performed a number of implantation experiments on rabbits. A lump of muscle from the thigh was grafted into the subcutis. The period of observation varied between 3 and 15 days.

Microscopic examination. All the preparations are made up of a central part consisting of the implanted tissue and a peripheral area of young mesenchymal tissue. If we follow the fate of the muscle graft, we observe clearly after only 3 days how the vitality abates. The nuclei become pycnotic, the striation is missing or indistinct. Nowhere in the grafted muscle fibres do we find any proliferative activity. In preparations taken after a longer period of observation, we have roughly the same situation. The muscle fibres lie like structureless chords. In the superficial layer of the graft we see some signs of disintegration in the later preparations. A little way in among the lifeless fibres we meet here and there accumulations of common non-specific granulation tissue. On the actual boundry, facing the surrounding connective tissue, we find the same histiocytic reaction tissue growing in large masses around the notched muscle fibres. Some fibres lie like loose fragments in the phagocytizing granulation tissue. No proliferative growth can be observed in this lifeless disintegrating muscle tissue. Quite a different picture is presented by the tissue which surrounds the graft like a capsule. In the earliest preparations, the capsule consists of young mesenchymal tissue with a rich accumulation of young capillaries. The reactive accumulations of cells are still relatively rare, and there is no specific growth of new tissue. In a 9-days old preparation, the mesenchymal tissue has differentiated to a considerable extent into common connective tissue, relatively poor in cells. The reactive non-specific cell accumulations have increased considerably and lie like a border layer around the surface of the graft. Outside in the connective tissue, we find here and there newly grown musculature in the form of more or less elongated spindle-shaped corpuscles. In some places we can find even rather large accumulations of such muscle spindles. With even greater magnification it is clear that the individual muscle spindles are always surrounded by mesenchymal tissue. On examining the boundry between these muscle-spindles and mesenchymal tissue, we can also see how the thin muscle fibres fringe into the mesenchymal network. In the older preparations we have the same picture with young musculature growing in varying quantities in the capsule, generally rather far away from the lifeless graft. The young muscle rudiments have now assumed a more elongated type of thin fibres, which gather together in greater and greater bunches.

Grefberg has studied in detail the question of the regeneration of musculature after transplantation, and has paid special attention to the fate of the graft during the first few days after transplantation.

He took small lumps of muscle from the thigh of rabbits and implanted them in the omentum of the same animal. The period of observation was 1, 2, 3, 4, 5, and 6 days, and up to two or three weeks. He was able by these experiments to confirm the accuracy of my observations. The graft soon became necrotic, and was gradually resorbed like a foreign body. On the fourth day young muscle fibres arose outside in the new mesenchymal tissue formed on the site of implantation. *Grefberg* could observe no morphologic connection between the new muscle rudiments and the grafted musculature.

In order to improve the method still further so as to get a more marked difference between the graft and the regenerated tissue, *Grefberg* used special methods of staining. He prepared the lump of muscle with trypan-blue before implantation in the omentum.

Trypan-blue is a semicolloidal dye, insoluble in lipoids, and in vital staining it stains only the non-cellular substances in the living cells, i. e. newly formed granules and spaces in the protoplasm (*von Möllendorf*). According to *Ries* living cells are stained only in exceptional cases, whereas dead tissue is easily coloured diffusely. *Strassman* considers that the state of degeneration of a tissue can be judged by staining at an early stage, when no morphological signs are visible.

The musculature was placed in a 1 % solution of trypan-blue in water for 1 minute, after which it was discoloured in sterile physiologic



Fig. 5.

Muscle-implantate in omentum. Survey of the boundary between the graft and the surrounding reactive tissue.

- a) the graft.
- b) histiocytic cell reactions.
- c) young muscle rudiments growing in the mesenchymal tissue.



Fig. 6.

The same prep. as fig. 5 in greater magnification. Histiocytic reactions around the graft.

salt solution for an hour, and then grafted immediately into the omentum. The period of observation was the same as in the preceding experiment. The fixed preparations were stained by Domagk's method with Kernechtrot-Aniline-blue-Orange. In these experiments the muscle graft was after 24 hours diffusely stained with blue. The grafted musculature retained its blue colour throughout all the series with increasing period of observation. After 4 days one could observe how small, more or less oval, homogeneous corpuscles, uniform in size, appeared outside in the young mesenchyma tissue, which was newly formed on the site of implantation and which surrounded the graft like a capsule. As a rule they were not stained, or had at the most a slight violet-blue tinge. Fig. 5 gives a picture of such a preparation, 6 days old. We see first the blue-stained graft (a), then a border zone consisting of histiocytic cell reactions (b), outside this is the layer (c) consisting of young muscle rudiments in common mesenchymal tissue. The original graft is composed throughout of dead musculature, entirely in a state of disintegration. We can see not the slightest signs of proliferation. Only a few odd leucocytes or histiocytes are found between the dead muscle fibres. Fig. 6 shows a greater magnification of the border layer between the graft and the surrounding areas. We can see all the irregular histiocytic cell reactions, and how the phagocytic activity is taking place in the superficial layer of the lifeless muscle graft. Here and there we see multi-nuclei cell forms containing

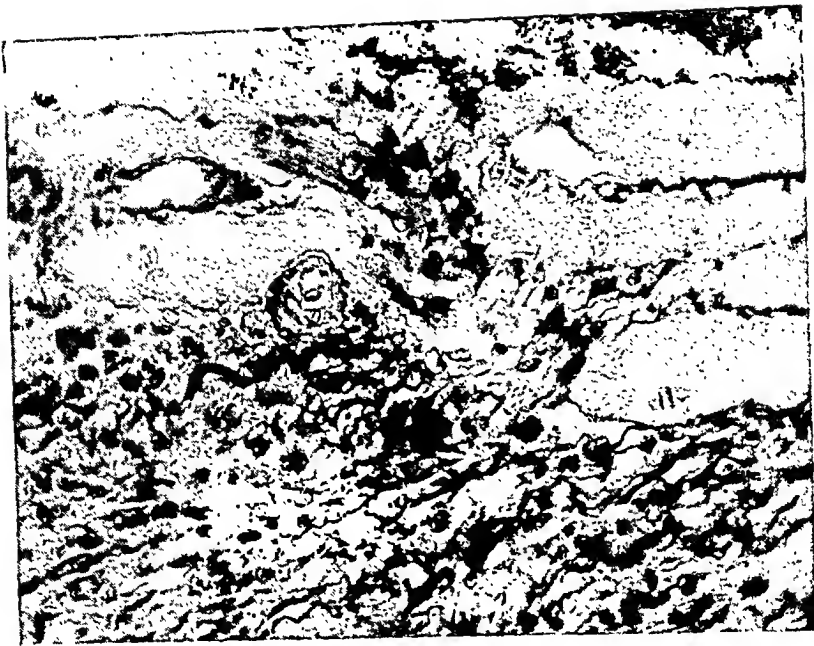


Fig. 7.

The same prep. as fig. 5. Young muscle rudiments growing in the mesenchymal tissue.

granules stained blue, to a greater or lesser extent. No signs of proliferative growth of living musculature is to be seen in this part of the preparation. If we shift the field of vision peripherally, we find an entirely different picture — see Fig. 7. The irregular histiocytic cell reactions cease, and instead we find a tissue exactly resembling common embryonic mesenchymal tissue in various stages of development. In this young blastemic tissue lie scattered the homogeneous corpuscles mentioned above. They are grouped in an irregular but characteristic manner, which clearly shows that what is happening is a development of an organized tissue. On closer examination, these small oval structures are seen to have a distinct longitudinal fibroid design, and their ends diffuse into the mesenchymal network. It is as though they were fringed into the surrounding medium. In some places we can even see traces of striation. The nuclei are relatively big with finely ramified chromatinic nets. These formations are almost crystal clear in the Domagk-stained preparations, and have as we have already stated only exceptionally a slight violet-blue tinge. This lack of colour is in sharp contrast to the markedly deep blue tone of the lifeless graft. As the period of observation is prolonged, the oval corpuscles become more and more elongated, and change into typical muscle fibres in the fully-developed muscle. It is thus evident that the first oval corpuscles in the earliest preparations must be considered to be the primary rudiments of regenerating musculature. Moreover, they sug-

gest highly the primary muscle rudiments in the earlier stages of embryonic development in, for example, the musculature of the extremities.

Discussion. The most interesting thing about these trypan-blue experiments is the obviously marked difference in colour between the dying graft and the regenerated musculature. Even after only 24 hours the graft is powerfully stained and retains this blue colour throughout all the series with increasing observation periods even to the stage when the graft consists of a single disintegrating structureless mass. As it is important to decide whether this blue colouration is due to the preparation with trypan-blue before implantation or to the aniline-blue during the Domagk-staining of the fixed preparation, Grefberg carried out control experiments. On examining a muscle lump immediately after preparation with trypan blue, it then appeared that only a peripheral layer of about one millimetre in breadth was stained. The central part was wholly undyed. It thus seems most likely that the blue colour of the graft is due to the staining with aniline-blue. Grefberg also injected trypan-blue into living musculature, which was then fixed in the usual way and stained by Domagk's method. It appeared that the living fully-developed musculature did not absorb the dye to more than a very slight greenish-blue shade. The connective tissue between the muscle fibres was, on the other hand, powerfully stained blue.

We ought to be able to draw the following conclusions from these experiments with trypan-blue. The fact that the muscle graft is stained blue indicates that the musculature dies at an early stage after implantation, long before there are any signs of regeneration. Domagk-staining shows that both newly formed and fully-developed living musculature either cannot be stained at all or only to a very slight blue shade. On the other hand, muscle grafts where we have positive evidence of death — the nuclei have disintegrated and the design is totally effaced — become clearly and powerfully stained blue after 2 weeks. The fact that the musculature can be stained by Domagk's method ought, therefore, to signify that the graft is dying or its vitality abating. Moreover, the diffuse staining with typan-blue of the superficial layers of the graft shows us that these layers are already dead at the moment of implantation. The newly formed musculature which regenerates 4 days after implantation is totally, or almost totally, undyed, and has all the characteristics of young embryonic muscle rudiments. This difference in susceptibility to staining does not, therefore, indicate any direct morphologic connection between the muscle graft and the regenerated muscle tissue.

Le Gros Clark has recently made some interesting observations during regeneration in muscle tissue grafted »in situ«. He excised small lumps of muscle and grafted them again »in situ«. The period of

observation varied between 3 days and 3 weeks. 10 minutes before the preparation was removed, bromophenol-blue was injected intravenously for the purpose of studying the vascularization of the graft. After 3 days the muscle graft looked like »a yellow slough, and was entirely unstained in contrast with the blue colouration of the surrounding muscle«. A histologic examination showed »the grafted fragments to be composed almost entirely of dead muscle fibres devoid of nuclei and undergoing fragmentation. At the immediate surface of the graft, however, and also close to the cut margins, a few fibres, or portions of fibres, still survived where their vitality had presumably been maintained by their close relationship to capillary vessels in the surrounding tissues«. In many of these marginal muscle fibres basophilic granules and small granules of fat could be shown to exist.

When it is a question of deciding to what extent these superficial muscle fibres — which he says survive — are significant for the regeneration, *Le Gros Clark* writes that it is »difficult to determine how much of the regenerated tissue in the graft has arisen from surviving surface and marginal fibres of the graft itself, and how much by the ingrowth through the fibroblastic tissue of sarcoplasmic sprouts from the cut ends of the fibres in the muscle surrounding the graft«. — Even if — as was to be expected as a result of the method used in the investigations — we are not able to decide with certainty from where the regenerated tissue emanates, the results are, nevertheless, of interest here, as they show that a muscle grafted even under the most favourable nutritional conditions — as in a well vascularized muscle medium — becomes necrotic. Only the most superficial can possibly maintain their vitality. We should, however, view these last observations rather sceptically. It is naturally difficult to judge with certainty whether these living muscle fibres are — as *Le Gros Clark* maintains — part of the graft, or whether he has not perhaps confused them with the ingrowing material from the surrounding tissues. Even *Le Gros Clark* himself found signs of degeneration in many of the marginal muscle fibres in the graft.

Results in implantation and extract experiments.

A comparison between the results after implantation of different skeletal layers — both hard and soft — shows clear resemblances. The first change during the earliest stages is a reduction in vitality within the graft. This can already be observed after two days, and increases gradually so that after 6—7 days we can see that the soft tissues have been transformed into a structureless mass, in which the pyenotic remains of the nuclei lie scattered irregularly. Simultaneously with this diminished vitality in the graft, there occurs around it a lively new growth of a young tissue, rich in vessels. This granulation

tissue, as it is generally called, consists of different components. It is constituted partly of histiocytic elements of different kinds and with phagocytic activity. These are generally situated nearest the graft. By far the greatest part, however, consists of young so-called embryonic connective or mesenchymal tissue. We see grouped concentrically around the small newly formed capillaries, large nuclei lying in a medium rich in protoplasm merging without a sharp dividing line into the surrounding areas. We evidently have here a young newly formed tissue, rich in vessels, and with pluripotent faculties of development. Only after a certain period — varying between 4—6 days — do we observe in our experiments the appearance of specific tissue of the same kind as the graft. The great difference in vitality between the graft, on the one hand, and the lively reactive tissue, on the other, is thus obvious. This contrast is sufficiently explained by the wholly different physiological conditions which exist in the graft and its surrounding areas. First and foremost the nutritive conditions are quite the opposite. In a foreign medium, the graft cannot expect, at least not at the beginning, any nutritive support from its new surroundings. On the contrary, we must reckon with a certain resistance to undisturbed growth as a result of all the cell reactions and exudations which are swiftly mobilised in the new medium, and which by phagocytosis and dissolution try to remove all physiologically foreign elements. Consequently, during the first period the graft is left for its continued existence wholly to its own intrinsic powers of growth. We may now ask how great these are likely to be. Immediately a tissue is cut off from its nutritive supply, disintegrating ferments begin to operate. This autolysis sets in at the moment the circulation is interrupted and can very soon result in severe damage. This fact is well known, not least by surgeons. The application for too long a period of, for instance, an Esmarch rubber tourniquet in the control of arterial hemorrhage can lead, as is well known, to fatal sequelae. After only two hours — or even a shorter period — muscle necroses, causing the serious deformity known as Volkman's contracture, set in. In spite of the fact that in such cases we are able by removing the obstacle to lead the blood stream back along its physiological paths, the autolytic ferments have evidently only in the lapse of a few hours managed to accomplish irreparable damage. This clearly shows that a tissue which is wholly separated from its nutritive supply must have a very reduced power of growth, if it has any at all. Consequently, an implanted lump of tissue has to struggle against two kinds of resistance to continued growth, namely both the histiocytic and exudative process, and its own intrinsic disintegrating processes. On the other hand, it is difficult to imagine any factors directly stimulating to continued growth, i. e. an improvement in the normal bioplastic energy of the tissue.

When making a decision as to the probability of transplanted cell

material continuing to grow in the new medium, it seems to me important to stop and consider the real significance of the biological reactions which take place during an implantation of tissue. It seems to me that the old belief that a cell can continue to grow after transplantation — even under very unfavourable conditions — has been adopted too uncritically. From a general biological point of view, there is no basis for assuming that an interruption of the entire nutritive supply would cause such a rise in bioplastic energy that the cells would start proliferating by division.

A more detailed study of the graft preparations gives us every reason to doubt the correctness of the earlier interpretations of the fate of transplanted tissue. We can, of course, assume that if a tissue is to be able to continue to grow in the new medium, it must retain its vitality all the time from the moment of transplantation. Consequently, if it were conceivable for the tissue to start growing as a result of its own energy, surely it would be most likely to do so immediately after transplantation. But we have observed, on the contrary, that a graft already shows degenerative changes after two days, changes which rapidly increase. No regeneration can be observed during these early stages. It is only at a later stage that the first signs of regeneration are seen. If, for instance, a new growth of bone could arise as a result of transplanted bone cells continuing to grow in the new medium, then a bone cell can remain alive for 5 to 6 days under relatively unfavourable conditions. After this period it must suddenly develop considerable additional growth energy so as to produce the lively growth shown, for example, in Figs. 1—2. This is, however, highly unlikely, especially as we can actually see in the early stages how the transplanted tissue undergoes an involution. When the osteogenesis gets properly underway, we can observe how a likely new growth of bone can take place in an extremely short period. This is clear from series of implantation experiments with varying periods of observation. In experiments with an observation period of 3—4 days we cannot see any signs of osteogenesis, the only bone tissue present is the transplanted tissue which has degenerated. Only the fifth or sixth day do whole fields of rapidly growing young osteoid tissue emerge. This certainly shows that a new growth of bone can clearly take place in a very short time. But should this rapid bone formation emanate from the transplanted material, we might reasonably expect the regeneration to have started at a considerably earlier stage — as early as the first or second day when the vitality of the graft is at its height. The longer the osteogenesis is delayed after the moment of implantation, the greater is the risk of the autolytic ferments becoming predominant. It is of a certain importance to prove the presence of this so-called latent period — from the moment of implantation up to the stage when the bone formation begins — which recurred regularly in every implantation.

A priori we cannot, of course, eliminate the possibility that the transplanted tissue can come into contact with the surrounding vessels, and thus, at a later stage, regain the vitality which clearly diminishes during the first few days after implantation. Such a reaction, however, could only explain how a tissue regains or conserves its vitality. Contact with the blood vessels of the new medium does not, on the other hand, explain the tissue growth as such. In other words, a vascularization from the surrounding areas only means that we have again, fully vital, the tissue we implanted. Consequently, it is not a question of the regeneration of new tissue, but only of the conservation of the vitality of the old. The revascularization is only a condition for the growth of a tissue, not a mechanism explaining the growth itself. A more detailed study of the histogenesis in bone formation ought, however, to give an answer to the question as to whether a revascularization — possibly combined with a revitalization of a somewhat degenerate tissue — or a regeneration, i. e. the formation of totally new tissue, really takes place.

If we follow the histogenesis of the young bone, it is obvious that they have a direct connection with the mesenchymal tissue newly formed on the site of implantation. As has recently been most carefully described by Häggquist, we can follow all the different stages typical of embryonic development of cartilage and bone from the embryonic mesenchymal blastema. We see how the nuclei collect together in more and more dense layers in the mesenchymal blastema. Around the nuclei we observe a brighter zone — endoplasmatic, according to Häggquist — while at the same time the nuclei themselves change their originally often triangular shape and become more rounded. In the substance outside this endoplasmatic zone — called by Häggquist the exoplasm — typical stabilizing ingredients are precipitated. All these different phases of development of bone tissue from a mesenchymal prestadium could be followed without difficulty in all our preparations in which it was possible to catch the osteogenesis at an early stage.

This typical histogenesis of bone is observed independently of the kind of graft used. The mesenchymal growth zone, hard bone and bone marrow, all stimulate a homogenous blastema into forming bone in the same way. It is also hard to believe that in a transplantation of, for instance, hard bone, the bone cells embedded in lime could possibly divide and proliferate into the surrounding areas. There are also no signs that the content of the haversian canals in the hard bone take part in the bone formation. If any bone-forming cells were present in the bone canals, one would reasonably expect to find young bone growing out in a broom-like fashion from their orifices at the surface of the bone. Such, however, is by no means the case. Instead, the connective tissue of the canals also becomes necrotic and the lumen becomes somewhat wider. It is also difficult to imagine that trans-

planted bone marrow cells could give rise to a mesenchymal bone formation, since bone marrow does not normally contain any typical tissue of this kind. Morphological analysis of different layers of the graft preparation thus shows unmistakably that the bone formation occurring after a transplantation cannot be due to a survival of the transplanted bone cells and their further growth in the new medium. We have in these experiments clear cases of genuine *regeneration* of bone tissue from a non-specific mesenchymal blastema, and not merely revascularization of the transplanted tissue.

This interpretation of the graft experiments is also confirmed by the *extract* experiments. These show that a factor able to activate the pluripotent mesenchymal tissue into forming cartilage and bone can be extracted from bone tissue and bone marrow. On the site of the extract-injections we can follow all the typical stages of the formation of cartilage out of a non-specific blastema. This histogenesis thus shows that what is taking place is a differentiation of newly formed tissue, and not a growth of cell material accidentally contained in the injected fluid. Such an assumption is also contradicted by the method employed, using concentrated alcoholic solutions for the extraction. Even the fact that we can fractionate the extracts in vacuum down to dryness argues against the assumption that the regeneration might be due to living cells accompanying the injection. The question as to whether the cartilage and bone formation is a specific reaction due to the extract can only be answered statistically, since positive results were obtained in 2—4 % of the control experiments. The fact that we can get bone formation after injection a non-specific stimulus does not necessarily argue against the theory of a specific bone-forming substance. The exceptional cases of bone formation in the control experiments ought merely to be compared with the rare heterotopic or idiopathic bone formation which can be found in any part of the body where, for some reason, a granulation tissue is formed. The marked tendency towards bone formation in different parts of the urinary tract often occurring after various experimental interventions is well known. I have emphasized elsewhere that this bone formation arises from the excretion with the urine of a specific osteogenetic substance. We may then assume that the substance in question also exists in the blood stream. Suitable mesenchymal media anywhere in the body can, for this reason, become under favourable conditions the starting point for bone transformation without the bone tissue necessarily emanating from some preexisting specific osteoblasts. *Annersten*, who worked up his material statistically states that it may be taken as proved that the positive results obtained with bone extract must depend on a factor present in these extracts, but absent in the control experiments. The other results also show a statistically established difference between the extract and the control experiments. Consequently, the extract experiments show the existence in bone tissue and marrow of a sub-

stance extractable by alcohol and able to activate pluripotent mesenchymal tissue into forming cartilage and bone.

We have already emphasized how difficult it is to draw any genetic conclusions from morphologic observations. All the investigations on the regeneration of musculature based, for example, on incision experiments are, therefore, of scarcely any value, as the different reaction phenomena lie so close to one another, that it is not possible to distinguish them clearly, even with the greatest magnification. Conditions are quite different, however, in the injection experiments. Here the preformed and the new musculature are often rather widely separated. In addition we can see the old musculature degenerating through the action of alcohol. All these phenomena indicate unquestionably that no morphological connection exists between the old and the newly formed musculature. But one naturally desires further proof of the truth of these suppositions. A detailed study of the muscle implantations ought, therefore, to provide a valuable supplement to the injection experiments.

Le Gros Clark's experiments with implantations »in situ« are of interest since they show that even under the most favourable conditions, such as those prevailing in a well vascularized muscle medium, by far the greatest part of the graft dies. This is clearly shown both by injection of bromo-phenol-blue and by microscopic examination of the graft preparations. It is, according to *Le Gros Clark*, only the most superficial muscle fibres on the surface of the graft, which, thanks to contact with the capillaries of the surrounding tissue, manage to preserve their vitality. He states that these most superficial muscle fibres contain, however, both fat and basophile granules indicating a certain degree of degeneration. Because of the method used for these implantation experiments »in situ«, it is of course, as we have already mentioned, difficult to decide with certainty whether the regenerated tissue emanates from the graft or from the surrounding areas. From the wounded surface of the incision in the musculature, a regeneration, of course, soon appears. If the tissue really can, as *Le Gros Clark* asserts, preserve its vitality by contact with the ingrowing capillaries, this nevertheless only means that the transplanted tissue reappears in the same degree of maturity as it had at the moment of transplantation. On the other hand, there is no reason to assume that a tissue, after achieving new contact with vessels, should acquire an additional amount of bioplastic growth energy. So that even if *Le Gros Clark's* observations are correct, he has only shown that a tissue may possibly be able to live in a new medium and still preserve the same degree of maturity it had before the implantation. The question of regeneration itself is, however, left unanswered.

Grefberg's experiments with trypan-blue make an interesting comparison with those of *Le Gros Clark*. Here the experimental conditions are quite different. We know that the superficial layer of the graft is

stained by the trypan-blue at the very moment of transplantation. This means, in all probability, that the superficial muscle fibres are already dead. If we then observe day by day the implantation prepared with trypan-blue, it is clear that a growth does not emanate from the superficial layer. Neither does any proliferation occur in the deeper muscle layers. In the contrary, we see that a powerful zone of histiocytic granulation tissue forms rather soon on the very border-line between the graft and its surrounding medium. During the 3 days immediately following implantation we have roughly the same picture. There forms, little by little, around the implantate a peripheric layer consisting almost entirely of mesenchymal tissue, rich in vessels, and further in, a histiocytic reaction tissue affecting the superficial layer of the graft and phagocytizing on it. We observe how the superficial muscle fibres become notched and irregularly hollowed out. All changes in this superficial layer are regressive. We search in vain for any proliferation activity in the form of plasmodium shaped muscle fibres. Only on the fourth day do new muscle fibres begin to appear. They then arise outside in the vascular-mesenchymal tissue, and have no contact with the old implanted musculature. In the stages when these young muscle rudiments begin to appear the phagocytosis has increased further and the central muscle graft has changed into a disintegrating mass totally without nuclei. It is evident that the primitive muscle rudiments have a totally different shape from the fully-developed muscle. These first rudiments outside in the mesenchyme are more or less round or oval formations with large central nuclei with a richly ramified chromatinic network. Reminding us, therefore, exactly of muscle rudiments in the embryonic stage. It is evident that we have in these implantation experiments, muscle regeneration in which the young muscle fibres pass through all the various phases to be seen during embryonic development, i. e. that we have here cases of genuine regeneration of musculature from a primary non-specific rudiment, and not a re-vascularization of an already fully-developed musculature. If it were only a question of revascularization of the superficial fibres of the graft we should, of course, get an entirely different picture than those given, for instance, in Figs. 5—7. The growth zone itself ought then to have been situated in the superficial layer of the graft and not outside in the peripheric mesenchymal tissue. The newly formed muscle fibres ought also to have been different in appearance. If it is only a question of revascularization, we ought to find musculature in the same stage of development as the transplanted one, i. e. fully-differentiated tissue. But as we have seen that the newly formed musculature is typically embryonic in appearance, then the fully developed musculature must — if we adhere to the revascularization theory — have the power to revert into a lower stage of development. In other words, the surviving muscle fibres would lose their striation, the nuclei would grow in size and move towards the centre, and the spindle-shaped long muscle

fibres would be reduced to a more or less oval shape. All this involution ought to take place in the superficial layer itself or in its immediate vicinity. Knowing the great sensitivity of musculature to an interruption of its blood supply, we may assume that this involution would occur within a short time after implantation. With every hour that passes after implantation, the chances of revascularization taking place are reduced on account of the activity of autolytic ferments and the phagocytes of the surrounding areas.

Comment.

It is clear from the above experiments that a connection must exist between the graft and the regenerated tissue, but that this connection cannot be explained by a direct spreading of the cells of the graft into the surrounding areas. The extract experiments with bone tissue and marrow show that there is every reason for thinking that the connection is regulated by certain chemical substances which can be extracted by alcohol. In order to understand these reactions better, it may be advantageous to make certain comparisons with embryonic development.

It is well known that the two theories attempting to explain embryonic development — preformation and epigenesis — have always been opposed to each other. Whereas the former considers ovum to represent a greatly diminished copy of the fully developed organism, and the development mechanism itself thus to represent merely a growth of various small parts, the epigenetic theory allows of the possibility that various parts may in the course of development be altogether newly formed and further developed. An important contribution towards the solution of this ancient question has recently been made by Spemann and his school. Spemann has shown by his experiments that we must expect the presence of epigenetic development. Spemann also ascribes a certain role to preformation but it is mainly the epigenetic mechanism of development that is of interest here. Spemann's contribution to causal embryogenesis is in trying to combine the two mechanisms of development, and show that both are active, though during different stages of development.

By his ingenious microsurgical isolation and transplantation experiments on Triton-embryoes, Spemann has been able to show how certain tissues are able to alter in character and are differentiated in a direction other than the original one. Thus, for example, a piece of presumptive abdominal skin is, if it is implanted in the vicinity of the presumptive ocular region, also able to develop as inceptions for the eye. On the other hand, it was found that certain areas were determinative for the development and had the capacity to influence other material which came within their spheres of influence. In view of these different possibilities of development for different tissue systems, Spemann set down some fundamental definitions. By *potency* is meant the capacity of the cell to develop in one or more directions. The development of a cell in a certain direction is called *differentiation*. As development proceeds, the capacity of the cell to develop in different directions is diminished — it is *determined* and only able to develop in one direction.

The fact that a tissue can be influenced from without by certain factors

so as to be differentiated in accordance with these exogenic impulses in a definite direction, is called *induction*. The induction itself is divided into two systems: the action system, which is the active agent and determines the induction; and the reaction system, which is the receptive part and permits itself to be induced. *Spemann* found in his experiments that certain areas were determinative for the development, so called centres of organisation. All tissue implanted in these sites changes its character and is differentiated in accordance with the conditions in the new medium. If, on the other hand, such an *organizer* is transplanted, it is able to exercise its dominant influence even in the new medium, and to affect the material in accordance with its specific nature. There is a current view that induction ceases with the conclusion of embryonic development.

It is, of course, the induction mechanism itself that is of greatest interest here. A vast amount of labour has been devoted to the search for the ways in which induction takes place. *Spemann* discusses various possibilities, and mentions both chemical and physical factors. Since tissues which have died in various ways — drying, freezing or overheating — can still induce, he considers that it must be a question of chemical substances and not of any forceful influence. Attempts have also been made to ascertain the nature of these substances. But these investigations have led to very different results. While some investigators consider them to belong to the steroid group, others are of the opinion that they must be nucleo-proteids. It has been shown, furthermore, that inducing effects are obtained from purely chemical substances, such as various pure acids, synthetic fatty acids, muscle-adenylic acid, etc. It is also known that a specific differentiation is not necessarily confined to a specific cause. All these observations cause us to hesitate when trying to explain the mechanism of an induction.

If we compare the questions arising in embryogenesis and post-foetal osteogenesis, we find some striking similarities. We may speak in both cases of a doctrine of preformation, according to which in one case the original ovum contains on a greatly reduced scale all the organs and tissues arising at a later stage, while in the other the pre-existing osteoblasts of the periosteal theory represent all the requisite ingredients for bone generation. We have seen that the theory of preformation is unable to explain all the reactions during embryogenesis. In order to understand certain phenomena arising during foetal development, we must assume that an initially unspecific material is influenced by certain exogenic impulses — *Spemann's* induction. Similarly, morphological tissue analysis in the implantation of skeletal and muscle layers in a medium of soft parts indicates that the newly formed osseous or muscle tissue develops from an initially unspecific blastema, which must, therefore, be influenced in some way by a specific factor in order to be able to develop in a certain direction. The similarity between experimental results during embryonic life and, for example, implantation experiments in the finished organism, is obvious. The post-foetal graft corresponds to the embryonic organizer or activator, and the mesenchymal tissue newly formed on the site of implantation — which permits itself to be influenced by the graft in a specially differentiated direction — by the embryonic system of reaction. It has further been shown in both cases that tissue

extract, free from cells, can influence a tissue into a definite differentiation. Even the first attempts to investigate the substances which possess this inductive capacity pointed in the same direction — »Preliminary evidence that the primary organizer is steroid« (Needham) »Dass dem hier untersuchten knochenbildenden Stoff möglicherweise Lipoid- eventuell Steroidnatur zukommt, entspricht den von Needham bei der Induktion erhobenen Befunden« (Annersten).

The idea of induction naturally tends to make us adopt fresh ideas on tissue specificity. The specific characteristics of the cell are considered to be indissolubly united with the cell itself, so that, if this dies, nothing remains of its function. Now induction shows that a tissue can be stimulated by certain exogenetic factors, so that it changes its character in the course of development and is differentiated in accordance with these exogenetic stimuli. This means that a tissue need not have from the beginning the characteristics typical of a later stage of development. At least part of the specific component accrues in the course of development. We can, therefore, speak of a synthesis between a more or less unspecific inception and a more or less specific component which need not necessarily be confined to a pre-formed cell material. As soon as we assume that different cell systems can influence each other in a specifically differentiated direction, we must also assume that certain factors can be transmitted from one cell to another. In other words, we are able to draw the conclusion that the specific growth factor is, at least partially, able to exist extracellularly, and thus need not exist merely as living tissue.

If we apply this interpretation of the idea of induction to regeneration of tissue, we are able to explain the implantation and extraction experiments on the fully-developed organism. In an implantation of tissue the graft dies, but the specific factor can be released from the cell and retain its properties even outside the original tissue. The same is true of an alcoholic extraction of a tissue. A strong alcoholic solution fixes the protoplasm with the result that the tissue dies and further growth in the form of cellular proliferation is not easily imagined. On the other hand, the specific factor can be extracted from the tissue, and exercise its function outside the latter.

The fact that bone tissue influences its surroundings in such a way that the latter can develop into the same tissue, must be fundamentally parallel to embryonic induction. I therefore considered it suitable to employ the term induction for the basically identical postfoetal reactions also. Bone regeneration takes place in accordance with an *induction mechanism*. The specific bioplastic energy of the original bone tissue is transmitted to the surrounding areas in the form of a substance, and not as a direct cellular spreading.

The idea of the induction mechanism conflicts, of course, with the prevailing theories regarding the genesis of growth within the fully-developed organism. The tissue growth in the implantation experi-

ments according to current opinions, is thought to result from a partial survival of the most superficial cell layers and their continued growth in the new medium. This view is thus based on two assumptions: a) that a tissue can come in contact with new blood-vessels in the new medium, and b) that further growth takes place by cell division of the thus revascularized tissue. According to this mechanism we are dealing all the time with fully developed tissue, so that we cannot, in the strict sense of the word, speak of a regeneration. What happens is a continued growth of the transplanted fully-differentiated tissue elements. It is, however, hardly conceivable that the fully-developed tissue should pass through an involution towards an embryonic prestadium. As has already been stressed, this would mean that muscle tissue, for example, would lose its striation and that the long spindle-shaped fibres would contract into a more oval shape. Further, the hard bone tissue would be subject to decalcification and develop in the direction of young embryonic mesenchymal tissue. Such a de-differentiation seems, however, very unlikely.

The induction mechanism for tissue regeneration is based, on the other hand, on entirely different conceptions than the revascularization and cell-division mechanism. Since a tissue can, as regards specificity, be divided into two components, we may adopt a new attitude towards the question of regeneration. Genetically speaking, the tissue is not an indivisible unit. We must take into account the existence of a more or less specific factor regulating or determining the special nature and function of the tissue. When investigating the causal factors of regeneration we are thus faced with two problems, firstly to explain the origin of the actual living mass of tissue from which the new tissue emanates, and secondly to elucidate the nature of the factor which influences this mass into developing in a specific direction. The induction theory can only explain the fact that an originally more or less non-specific tissue develops in a certain direction.

For complete understanding of all the reactions connected with tissue regeneration we must obviously be able to explain both the origin of the primary blastema and the specific differentiation. At a tissue lesion we see how a granulation tissue, rich in vessels, rapidly forms. Several layers of large nuclei group round the small lumina of the capillaries, and between these young vessels a syncytium exists, rich both in protoplasm and nuclei of the same appearance as the endothelium of the capillaries. This tissue is well known under various names. One of these is vascular mesenchyme, which seems highly suitable as it emphasizes the close connection between floating and organized tissue. It is obvious that this tissue is the origin of the blastema out of which the specific differentiation later takes place. In other words, it contains omnipotent or pluripotent faculties of development. The direction in which the development takes place is not, however, determined by the tissue itself, but by exogenic factors. If

this pluripotent tissue does not in its early stages receive some exogenous stimulation towards differentiation, it forms common non-specific connective tissue. We do not know anything at present regarding the origin of the young pluripotent tissue. We can only state that this mesenchyme with large nuclei embedded in a succulent protoplasmatic mass appears at every tissue lesion. We cannot, of course, explain its origin merely by cell division, as this presupposes the existence of a tissue those cells can grow by division. Here it is a question of explaining the origin of a mass of tissue in sites where no similar tissue previously existed.

Summary & Conclusions.

When investigating the causal aspect of a healing process, the first and most important question is to decide whether the regeneration emanates solely from the old tissue or whether some other material may also play a rôle. The most suitable method for settling this question is implantation. It is of the utmost importance here to study the implantation preparations very carefully during the first few stages immediately after transplantation.

Implantation of the various skeletal layers — the mesenchymal growth zone, hard bone and bone marrow — showed that the graft dies shortly after transplantation, i. e. within 2—3 days. New bone is not formed until 4—6 days after implantation. This osteogenesis arises through a differentiation of the mesenchymal tissue which forms on the site of implantation.

Since it is clearly not a question of direct cellular proliferation from the graft into the surrounding medium, the author explains the osteogenesis by assuming the existence of a substance which is liberated from the bone tissue and bone marrow, and which passes over into the surrounding medium where it activates the newly formed pluripotent mesenchymal tissue into forming bone. That such a substance actually exists has been proved by experiments with alcoholic extracts — totally free from cells — of bone tissue and bone marrow (*Levander, Annersten, Bertelsen, Lacroix, Willstaedt*).

The author considers that striated musculature also regenerates from a mesenchymal blastema. After injection of alcohol into musculature, a rich growth of mesenchymal tissue occurs around the musculature which is damaged by alcohol. In this mesenchymal medium grow young muscle rudiments which have no apparent connection with the original musculature. After implanting muscle, one can observe that the implantate dies and that the new musculature is differentiated out in the surrounding mesenchymal tissue.

The author compares these reactions, where an originally non-specific mesenchymal tissue differentiates in a specific direction, with

embryonic induction. Induction would mean in the regeneration of tissue that a factor, which is necessary for the specific differentiation, can be liberated from a tissue and pass over into the surrounding medium where it induces a pluripotent mesenchymal tissue in a specific direction. There is every reason for thinking that this factor, will ultimately be identified as a definite chemical substance.

The theory of direct cellular proliferation does not, for example in implantation experiments, give us a regeneration mechanism in the true sense of the word. It only states that certain cells can maintain their vitality after transplantation and continue to live in the new medium. According to the induction theory, on the other hand, the process is really one of *regeneration*, i. e. an entirely new material is formed in the fully-developed organism independently of the actual mass of old tissue. This means that even in the fully-developed organism, differentiation of an originally non-specific medium takes place just as in embryonic development. In other words, regeneration is a repetition of the reactions which take place during the foetal stage. Induction explains only the specific differentiation. We still do not know how the actual mass of tissue arises during regeneration.

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PERIARTERITIS NODOSA A CASE, PRESUMABLY CAUSED BY SULFONAMIDES*)

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Periarteritis nodosa (Kussmaul & Maier 1866) has been recognized in the Scandinavian countries by Harbitz in 1917, but it is only in more recent years that a greater number of cases has been reported.

Since 1930 nearly 30 cases have thus been reported from Sweden (Herlitz 1930, Gjertz, Nordlöv & Svenmar 1939, Bergstrand & Tisell 1939, Westergren 1940, Sköld 1942, Henschen 1942, Svanberg 1944, Oldfelt 1946), from Finland a couple of cases (Lindberg 1931, 1932), and from Iceland one case (Dungall 1936).

From this country there are two clinical cases (Banke 1941, Heinild 1942). Moreover, some cases have been reported in which vascular lesions of a similar type have been observed. Thus Eskelund (1942) has described a case of pulmonary sclerosis which has been interpreted as periarteritis nodosa. Heerup has found periarteritis nodosa-like changes in a case of leukemoid eosinophilia (Engbæk, Heerup & Thomsen 1942), and Teilum (1946) has in lupus erythematosus disseminatus found changes of the periarteritis nodosa type.

The etiology and pathogenesis of the condition was for a long time obscure. The earliest mechanical theories, just like the assumption that it was a special form of syphilis (Versé 1907) had soon to be abandoned. Also the presumption that the affection was caused by specific virus with a special affinity for the vessels, proved not to be tenable, even though some few authors thought that they were able to transfer the disease to animals (v. Hann 1920, Harris & Friedrichs 1923).

*) Read in a somewhat different form before the Funen Medical Association, 26th January, 1947.

In 1925 *Gruber* advanced the view that in all probability periarteritis nodosa would have to be looked upon as an allergic reaction on the part of the arterial system during the course of various infectious-toxic conditions.

This hypothesis has since been accepted by practically all pathologists. In support hereof the considerable resemblance has been stressed which exists between the changes in periarteritis nodosa and those encountered in experimental allergy, e.g., in Arthus phenomenon (*Gerlach* 1923) or in generalized allergic reactions (*Klinge & Vaubel* 1931, *Vaubel* 1932, *Apitz* 1933). *Rich & Gregory* (1943) have experimentally produced vascular processes which are identical with periarteritis nodosa.

Not infrequently periarteritis nodosa occurs during the course of, or associated with, allergic conditions, e.g. asthma (*Bergstrand & Tisell* 1939, *Rackeman & Greene* 1939) or rheumatic fever (*Herlitz* 1930, *Friedberg & Gross* 1934), and the latter affection is indicated to occur in the history of at least 10 per cent. of the cases.

Periarteritis nodosa, therefore, is now included among the allergic vascular diseases, according to *Rössle* (1933) in the group of »rheumatoid« diseases, as this author reserves the designation »rheumatic« for those vascular conditions which present granulomatous processes in the walls of the vessels, or in which these processes are combined with the rheumatic changes in the free connective tissue described by *Klinge* (oedema, fibrinoid necrosis, formation of granulomas).

As, however, the criteria set up by *Klinge* for rheumatic infection may also be encountered in other conditions (*Teilum* 1946, *Bergstrand* 1946), and as rheumatic fever will now have to be looked upon as an etiologic unity, inter alia because the etiological significance of the streptococci must now be considered to have been finally established, *Teilum* (1946) has proposed that a distinction be made between a genuine rheumatic infection (febris rheumatica) and a group of »pararheumatic« conditions, which has the pathogenesis in common with rheumatic fever, but a different etiology, and which includes lupus erythematosus disseminatus, an arteriolitis granulomatosa (allergica) described by *Teilum*, and periarteritis nodosa.

However, it has not so far been possible to furnish the conclusive proof of the allergic pathogenesis, the demonstration of an exciting allergen in man. Hitherto the allergen has generally been presumed to be of a bacterial nature.

The last few years have brought us essentially nearer to the establishment of this proof, as it has been possible to isolate a group of cases, which histologically has presented the picture that is characteristic of periarteritis nodosa, and in which the allergen — of a non-bacterial nature — has been known.

After the introduction of sulfonamides in the therapy the presumption quickly arose that certain of the undesired effects observed

on administration of these substances might be of allergic nature (Hageman & Blake 1937). By experiment it has been demonstrated (Schönholzer 1940, Davis 1942) that sulfonamides may be linked to serum proteins, and Wedum (1942) and Gerber & Gross (1944) were able to produce allergic reactions in animal experiments by means of such compounds. In man it has been difficult, however, to demonstrate hypersensitivity, both by direct cutaneous reactions and by passive reaction of hypersensitivity, but of late years the attempts seem to have been successful, as by employing serum from patients who had reacted for a second time to the injection of sulfonamides Schaffer, Lentz & McGuire (1943) have succeeded in obtaining a positive Prausnitz-Küstner reaction, and Leftwich (1944) has obtained positive reactions by means of a somewhat different technique. In these studies sulfathiazole has demonstrated a somewhat greater proneness to produce allergic reactions than have the other sulfonamide preparations, and the different preparations, according to Leftwich, show a certain specificity.

Histologically, the changes of the organs of patients who have died following sulfonamide reactions have often presented distinctly allergic features (French & Weller 1942, Merkel & Crawford 1942, Simon 1943, Black-Schaffer 1945, More, McMillan & Duff 1946, French 1946). The lesions have been of varying extent and intensity in the individual cases, and have been demonstrated in practically all the organs. It is a question, partly of vascular necrotising processes with oedema, fibrinoid necrosis of the vessel walls and their surroundings, often an eosinophilic cellular exudate, partly of focal, often fibrinoid necroses and granulomatous processes in the parenchyma of the organs and the interstitial connective tissue, and finally, of interstitial inflammatory processes without necrosis, that is, essentially changes which bear a close resemblance to those demonstrated in various forms of experimental allergy.

Black-Schaffer emphasizes that even though the changes which were present in the cases described by him did not, as regards quantity, compare with those seen in periarteritis nodosa, the fundamental features were identical to such a degree as to suggest a difference in the intensity and duration of the irritant rather than a difference in quality.

In 1942 Rich reported 7 cases of medicamentally provoked periarteritis nodosa. In one of these cases the patient in association with serum therapy had developed a violent attack of serum sickness which terminated fatally in the course of some weeks. Autopsy showed typical periarteritis nodosa changes. In 5 other cases the patient had been given serum as well as sulfathiazole, in the last cases only sulfathiazole had been administered. Necropsy or biopsy examination in all cases showed the characteristic vascular lesions. Rich considered it likely that the processes had been caused either by the serum or

the sulfathiazole. The conditions on account of which treatment was given being acute infections, they could not be reasonably presumed to have been of significance for the processes, and there was but slight probability of the patients having had the vascular changes previous to the treatment.

Later in 1942 *Rich* reported yet a case which seems convincing as regards the part played by the sulfathiazole in the development of the disease. The patient was operated upon for carcinoma of the scrotum. The diagnosis had been made on a biopsy 5 months previously, and neither the biopsy, nor the tissue removed at operation presented any vascular changes. For 3 days after the operation the patient was given sulfathiazole as a prophylactic measure. On the 8th post-operative day a conjunctivitis developed, accompanied by a rise in temperature, for which reason sulfathiazole was again given; the medication was continued till the patient died from uræmia on the 18th postoperative day. Autopsy revealed widespread vascular changes, closely resembling periarteritis nodosa.

A very similar attack following sulfathiazole therapy has been reported by *Lichtenstein & Fox* (1946), and *Rosenak & Maschmeyer* (1945) have described a case following treatment with sulfadiazine.

Thus there can be no doubt that sulfonamides may produce allergic tissue reactions, which in the severest cases may attain the picture which is characteristic of periarteritis nodosa.

Rich considers it likely that by being linked to serum protein the sulfonamides acquire the characteristics of a hapten, and thus act as an antigen. He considers that other compounds may have a similar effect, and in 1945 has described a case with periarteritis nodosa-like changes, which must be presumed to have been caused by iodine. Finally, *Gibson & Quinlan* (1945) have reported a case which arose following thio-urca therapy, and *Hill & Damiani* (1946) a case caused by the insecticide DDT.

Case Report.

The patient was a 20-year-old man. A paternal aunt had asthma, but otherwise there were no allergic diseases in the family. The patient had always been moody, dawdled away time, could not perform any work. His intellect was unimpaired. The last twelve months before admission he had suffered from asthmalike attacks. During the six months preceding admission he had moreover had sinusitis, for which, in the period from October 31st 1945 till admission, he was punctured 9 times, each time with application of alphasol (a sulfathiazole derivate); the quantity is not stated. As the condition did not improve, and as in the course of time he became too poorly for treatment in the out-patient department, he was admitted to the ear-department of the county hospital in Herning on December 4th, 1945. A bilateral maxillary resection was done on December 5th, which showed the mucous membrane to be highly polypous; unfortunately no microscopic examination was done. As on the 8th post-operative day the temperature was rising sulfa-

thiazole was given. 3 days later an exanthema developed, for which reason administration of the drug was discontinued, and the temperature slowly fell to normal. The quantity of sulfathiazole is not recorded, but a quantity of about 20 g has presumably been given.

As the asthma was aggravating the patient was transferred to the medical service December 28th. The temperature was again rising, the patient complained of pain in the throat and was somewhat dyspnoeic. Stetoscopy of the lungs showed signs of bronchitis (sedimentation rate 41 mm, hemoglobin 90 % (Sicca); differential count: 27 % eosinophile leucocytes). From December 31st »chemosept« (sulfathiazole) was given. Upon administration hereof the temperature rose still further (see fig. 1) while at the same time, January 2nd 1946, the patient complained of pain and tenderness of the lumbar regions, and a slight albuminuria developed (output of urine 800—1000 cc in 24 hours). Because of the high temperature (40.4°) and the negative findings by microscopic examination of the urine, the albuminuria was not given any attention. On account of nausea alphasol was substituted for chemosept on January 3rd. The same day the temperature began to fall; a maculated exanthema which had developed on the legs in the course of the day faded away within the next few days. January 7th hematuria was demonstrated, for which reason the administration of alphasol was discontinued after the patient had been given altogether 13 g chemosept and 30 g alphasol (= 90 g alphasol mixture). The next two days there was a pronounced hematuria and severe oliguria (150 cc a day), but by means of salt water given subcutaneously, sodium bicarbonate and sodium sulphate intravenously, and diathermy over the renal regions the secretion of urine was started again so that on the 11th January the output of urine was 1650 cc. Hereafter, however, an isostenuria, which had not been present previously, persisted throughout the rest of the course. While there was oliguria, the blood urea, as expected was increased (max. 123 mg %), in order thereafter to fall, not below 60 mg %, however; in the end the blood urea rose to 257 mg %. Among the other urinary changes may be mentioned that the albuminuria persisted during the rest of the course, the amount of albumin fluctuating considerably (Esbach: traces — max. 12 %). The hematuria declined considerably, but did not quite disappear.

Already with the onset of the oliguria the patient began to complain of diffuse, undefinable abdominal pains; it was not possible, however, by examination of the abdomen to demonstrate anything abnormal. In the following period these pains increased considerably so that the patient at times was greatly distressed, and opiates were necessary. During the attacks of pains he sought relief by taking up peculiar positions, either a ventral position, or hugging his knees with the pillow pressed against the abdomen. The stools became frequent of varying consistency, most often thin but they were without visible blood or mucus. Benzidine reactions were, however, positive, a few of them even very vigorously so.

The patient grew ever more poorly and increasingly stuporous. There came increasing muscular weakness, approaching complete prostration. He was constantly afflicted with nausea and slight vomiting (blood urea moderately increased, alkaline reserve and serum-chlorine normal, blood pressure about 130 systolic, 90 diastolic, serumprotein normal). For some time there was pronounced oedema of the face. Intermittently the patient had slight attacks of asthma, which were easily controlled by means of aleudrin. In the end of January the patient began to get joint pains and present objective changes of the joints in the form of swelling, restricted moveability and accumulation in wrist, knee and ankle joint, while at the same time disturbances of sensation (paresthesias, hypesthesias, abolished deep sensation) and changes of the reflexes of the upper and lower extremities developed.

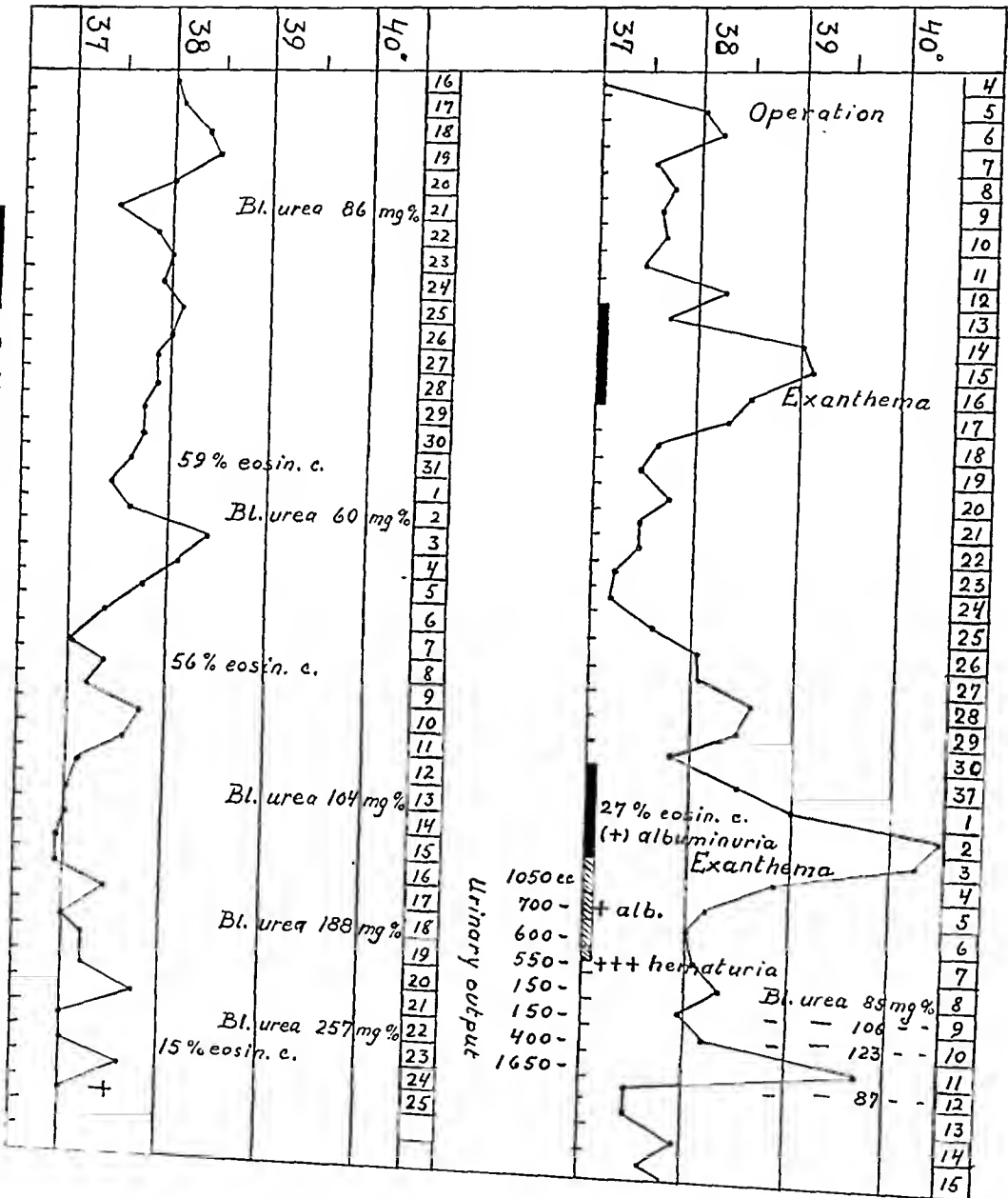


Fig. 1.
Temperature curve (Evening temperature).

The unusual abundance of symptoms associated with the strikingly vigorous eosinophilia (up to 59 %) which besides in the peripheral blood was demonstrated to be present to a very pronounced extent in the sternal marrow (eosinophile myelocytes 9 %, eosinophil polynuclears 44 %) made it possible to establish the diagnosis periarteritis nodosa with very great certainty. Further the peripheral arteries were found to be strikingly rigid, somewhat heterogeneous; still it was not possible to demonstrate isolated nodes on them with certainty.

At this time it was evident that the patient had not many more days left and 14 days after establishment of the diagnosis the patient died, uremic (February 25th, 1946, 12 weeks after admission).

Among the other examinations that have been done should be mentioned: spinal fluid: clear, 2/3 cells, 20 albumin, 2 globulin. Ophthalmoscopic examination: nothing abnormal. Antistreptolysin titer 220, Mantoux III +; Wassermann and gonococcal complement fixation tests were negative. Examination of the blood January 21st: hemoglob. 70 % r. blc. 4.1 mill., corpusc. vol. 28 %, wh. blc. 17,000 with 59 % eosinophil, reticulocytes 0.7, platelets 250,000; plasma color 6. X-ray examination of the lungs: Increased configuration of the lungs with finely punctate configuration caudally and medially on both sides, subsiding laterally. From the hilar region, which is not enlarged, streaks and stripes extend upwards towards both apices.

Autopsy.

Already at the autopsy examination it was possible to confirm the diagnosis with a fair degree of certainty.

The corpse was extremely emaciated with slight ankle oedemata. There were no cutaneous changes. *Knee and ankle joints* rather large as a consequence of the severe muscular atrophy; no definite swelling of the joints.

The *tonsils* were rather atrophic, without plugs or abscesses. The *larynx*, *trachea* and *main bronchi*, the *thyroid gland* and the remnants of the *thymus* were without changes.

The *pleurae* were smooth and glistening, no exudate in the pleural cavities. The *lungs* were voluminous, pale, emphysematous, with moderate hypostasis; no *pneumonia*. In the bronchial ramifications a small amount of mucus. Several sections exhibited no changes in the branches of the pulmonary artery.

The *heart* was not enlarged. The pericardium without changes, the epicardium presented a few, whitish tendinous spots. The coronary arteries were thick and rigid, slightly tortuous with striped and nodular thickening. The valves and the endocardium were without changes. In various places in the myocardium, especially in the lower part of the left ventricle, there were rather close, small whitish spots which seemed to be localized around thickened branches of the vessels. No clear changes in the muscle were noted, especially, there were so cicatricial changes.

The *great vessels*, the *aorta*, the *iliac*, *subclavian arteries* and the *carotids* were natural, macroscopically. The *oesophagus* and the *stomach* were without changes.

There was no ascites and no thickenings of the serosal surfaces. By examination of the *ileum* dark, necrotic areas to half-penny size were found, however, without perforations. In the mucous membrane there were several dark ulcerations pin-head to hemp-seed size, and besides, larger necroses of the mucous membranes corresponding to the changes found on the serosa. In the mucous membrane of the *colon* no necroses were seen.

The *pancreas* and the *adrenals* exhibited no changes.

The liver was of normal shape and size, the colour reddish-brown, the consistency not clearly altered. The cut surfaces of the right lobe in some places, and in the left lobe in several places, presented increase of the portal connective tissue, and it was possible to recognize cross sections of thickened or obliterated vessels. No changes of the parenchyma were in evidence. Gall-bladder and bile ducts were normal.

The spleen was slightly enlarged, with accentuation of the follicles. No infarcts. The abdominal lymph-nodes were not enlarged.

The kidneys were of normal size. The surfaces very mottled, with alternative lighter and darker areas. On sections severe changes were disclosed, with thick whitish stripes and streaks along the arciform and interlobular arteries. The configuration of the tissue was somewhat obliterated and mottled. In the left kidney a fresh, walnut size, infarct-like necrosis was seen which did not seem to extend quite to the surface. The pelves, ureters, bladder and prostrate were without changes.

One of the knee joints was opened; the synovial membrane was normal. The skull was not opened.

The diagnoses made at autopsy were as follows: — Presumably periarteritis nodosa, — infarct of the left kidney, — disseminated necroses of the ileum, — pulmonary emphysema and chronic bronchitis, — emaciation.

Microscopic examination.

Microscopic examination showed widespread and severe necrotising vascular lesions in practically all organs examined; thus *tonsils, heart, liver, kidneys, adrenals, spleen, pancreas, mesenteric vessels and intestinal walls, seminal vesicles, striated muscles and peripheral nerves*. The only organs in which vascular processes could not be certainly demonstrated were the lungs and the synovial membrane.

The processes were almost exclusively localized to the arterial system, comprising arteries ranging from the size of arterioles to about coronary

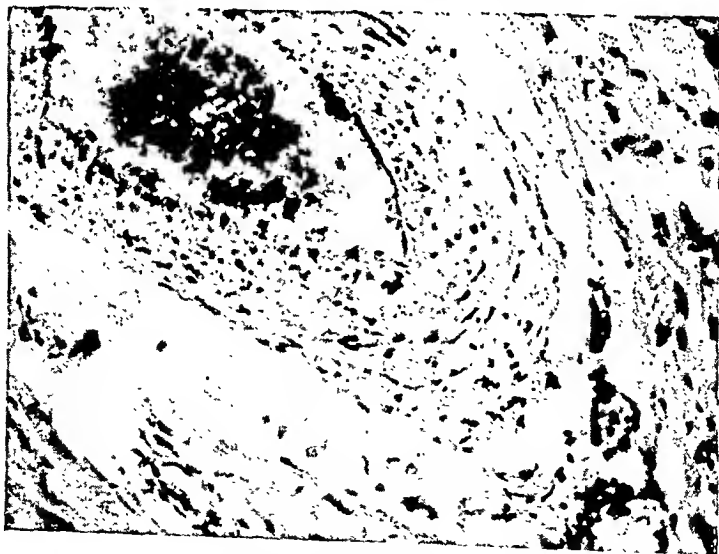


Fig. 2.

Oedema, vacuolization and beginning fibrinoid degeneration of the media in a coronary branch.

arteries. Only in some few places the veins presented changes. None of the larger arteries were affected, whereas occasionally changes were seen in the adventitial branches of the arteries, e. g., of the aorta.

The earliest changes consist in a disorganization of the muscle cells of the innermost layer of the media, with oedema, vacuolisation, a suggestion of fibrinoid necrosis and initial, very slight perivascular cellular infiltration (fig. 2).

Far more frequently a massive fibrinoid necrosis is found of the wall in the entire circumference of the smallest arteries (fig. 3); in the somewhat larger arteries often only part of it is involved (fig. 4). At the same time there

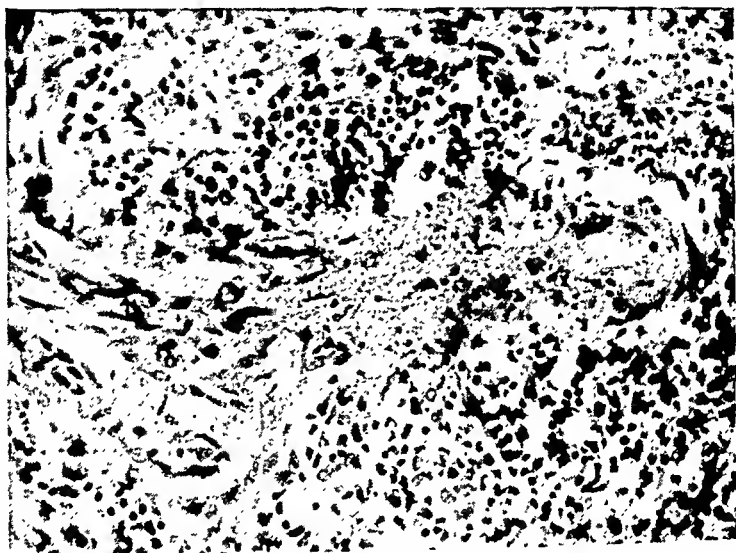


Fig. 3.
Fibrinoid necrosis of an arteriole in the kidney.

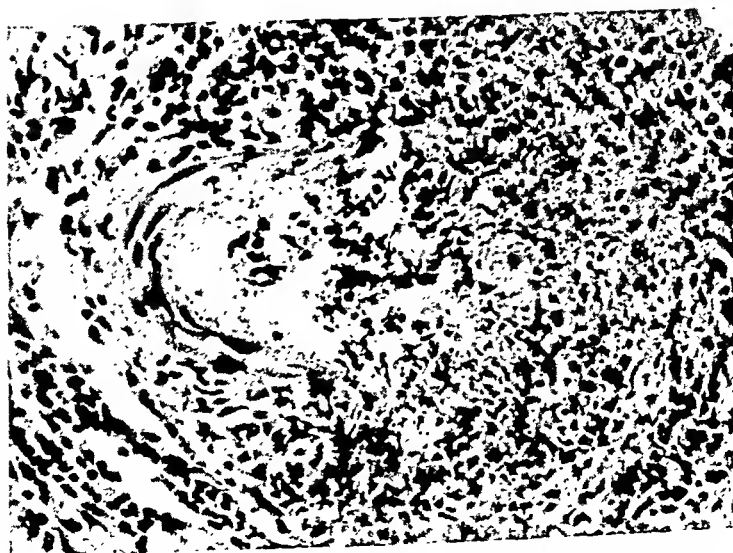


Fig. 4.
Partly destroyed small arteria in the suprarenal capsule.

are varying degrees of perivascular cellular infiltration with many eosinophil leucocytes. The cells of the intima are swollen, and in places there is sub-intimal cell proliferation.

In more advanced stages and in the somewhat larger arteries great fibrinoid masses are revealed, lying in a massive cellular exudate, and around the vessels there is a granulation tissue, extremely rich in cells, extending through the destroyed vessel wall. Moreover, there is frequently a considerable subintimal cellular proliferation, occluding the lumen wholly or in part. The fibrinoid masses may sometimes be calcified.

In the later stages the cell infiltration is diminished, and the granulation tissue is transformed into a partly hyalinised connective tissue with lymphocytes and phagocytic cells. Often remnants of fibrinoid masses still persist. The lumen is obliterated, or displays re-canalization (fig. 5).

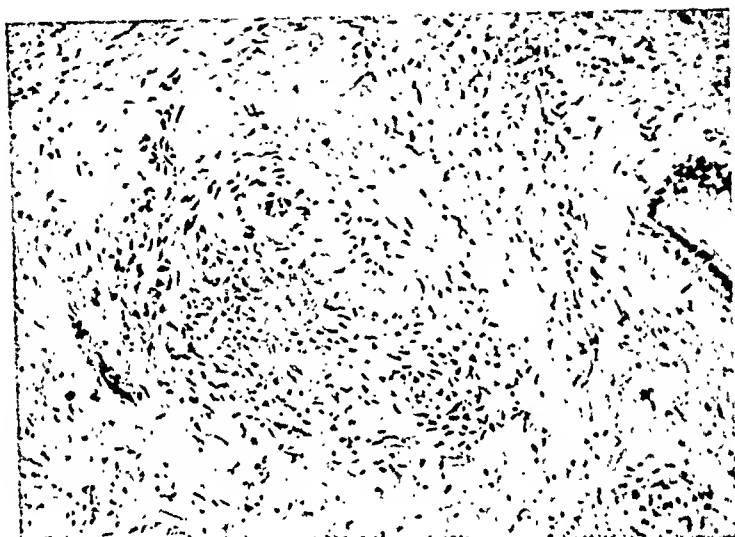


Fig. 5.

Healing lesion in the liver. Remnants of fibrinoid material in the center.

In the *myocardium*, in addition to widespread, active vascular processes a slight diffuse interstitial cell infiltration is exhibited of lymphocytes and histiocytes, and some few eosinophil cells. Here and there focal necroses of the muscle are seen, with initial proliferation of fibroblasts, but no connective tissue scars are in evidence.

In the *striated muscles* considerable degenerative changes are seen, with atrophy and homogenization of the muscle cells and considerable clustering of the nuclei. Moreover there is moderate oedema and slight interstitial cellular infiltration. Where no vascular changes are present, the picture may closely resemble the changes encountered in dermatomyositis.

In the *liver* the vascular processes are largely healing. In the portal spaces the connective tissue is increased, with infiltration of lympho- and histiocytes. Remnants are found of wholly or partly destroyed branches of the hepatic arteries, often surrounded by macrophages filled with pigment, and in several places there are still remnants of fibrinoid material. The vena portae branches seem in several places to be involved in the processes. The parenchyma itself presents a good deal of stasis, but no necroses and no granulomatous processes.

Abdominal pain	57 %	Purpura	22 %
Edema	52 %	Cyanosis	21 %
Loss of weight	48 %	Eosinophilia	19 %
Symptoms of neuritis	48 %	Nausea	17 %
Hematuria	47 %	Palpable nodules	16 %
Hypertension	46 %	Pain in the chest	16 %
Dyspnea	41 %	History of allergy	15 %
Emaciation	36 %	Convulsions	15 %
Cough	36 %	Icterus	12 %
Vomiting	31 %	Vertigo	8 %
Sensory involvement	31 %	Pos. ser. syphilis react.	8 %

As appears from the case history, our patient presented a very great number of these symptoms.

In the considerations of the etiology one cannot, in the present case, help getting an impression of a striking connection between the sulfonamide medications and the development of the disease. It will therefore have to be considered whether sulfonamide therapy may have played any part for the development of the disease, or whether it is merely a question of an accidental coincidence between the sulfonamide treatment and a pre-existing periarteritis of unknown etiology.

Concerning the possibility of a pre-existing periarteritis, the clinical picture may very well lead to the belief that such a lesion was present. The patient had always been moody and incapacitated for work, and during the last twelvemonth he had suffered from asthma-like attacks, and, later, sinusitis. It was, therefore, possible that he had had the condition for some years or, in any case, the last twelve months or so. Cases running such a protracted course and with very slight initial symptoms are not unknown (*Harris & al.*, *Fitz & al.*), even though most frequently the condition takes a more rapid course, terminating fatally within some months.

The microscopic examination, however, goes decidedly against such a view, as the processes found were very chiefly active, and only in the liver the processes were mainly healing processes, although, more frequently they were not quite cicatricial yet. With a wide allowance, all the changes may without difficulty have arisen within the 12 weeks elapsing from admission to the hospital till the occurrence of death, possibly in an essentially shorter time. If the condition had persisted beyond this period quite cicatricial changes must have been found to a far greater extent than was the case.

The possibility of a pre-existing periarteritis nodosa must therefore be considered extremely unlikely, and the condition must be assumed to have developed during the stay in hospital.

The assumption of the sulfonamides as the cause of the disease must be based on a sensitization to sulfathiazole. The possibility of such a sensitization has been afforded by the protracted treatment by applying alphasol in the maxillary sinuses, since alphasol in the or-

The adrenals show violent processes in the vessels in and around the capsules. In the cortex disseminated necroses are found, sometimes with calcification, and in one of the adrenals there is a large necrosis involving both the cortex and the medulla.

In the kidneys the vascular lesions are very widespread and severe, involving the large arteries as well as the arciform and interlobular arteries. In the arterioles it is only rarely possible to follow the changes right out to the glomeruli. Normal glomeruli practically are not found, they all present extensive hyaline thickening of the basement-membranes with splitting up of the capillary loops, or fusion and hyalinization of the whole of the glomerulus or parts of it. In some places remnants are found of fibrinoid necrotic material. Moreover, there are partial fusions between the hyalinized capillary loops and the capsule, but only to a very slight extent proliferation of the capsular epithelium with crescentic formations. The tubules are highly atrophic, others are dilated. Many of them contain casts, composed of eosinophil material with necrotic cells and leucocytes. Interstitially, there are considerable infiltrations of leucocytes among which many eosinophil cells are seen.

The lung tissue exhibits emphysematous alveoli, with slight oedema without cellular infiltration. The bronchi are often contracted and contain mucus. There is mucous metamorphosis of the bronchial epithelium and hyaline thickening of the basement-membrane. Moreover, there is some lymphocytic infiltration of the bronchial wall. Vascular or granulomatous processes are not observed.

The microscopic examination thus has demonstrated widespread changes of the arteries of the type characteristic of periarteritis nodosa. The changes are mainly fresh and active, and only to a small extent they are healing, essentially in the liver. Besides, disseminated interstitial inflammatory processes have been found in the myocardium and the kidney, and necroses in the suprarenal glands.

Discussion.

The case here presented must be considered a typical case of periarteritis nodosa combined with asthma from a clinical as well as a pathologic-anatomical point of view, and it is likely that the sinusitis which led to hospitalization was also of allergic nature.

The combination of asthma and periarteritis nodosa occurs not very infrequently and was found by Rackeman & Greene in 27 out of 229 cases. It is emphasized by Bergstrand that in cases of this kind the lungs often present vascular changes. It has not been possible to demonstrate such changes in our case.

Otherwise, the polymorphism so important for diagnosis and so characteristic of the disease, has been very pronounced in this case. Harris, Lynch & O'Hare (1939) on the basis of 101 cases indicate the following frequency of the different symptoms:

Fever	80 %	Headaches	29 %
Leucocytosis	70 %	Arthritis	27 %
Albuminuria	65 %	Atrophy	25 %
Rapid onset	58 %	Visual disturbance	23 %

ganism is decomposed and secreted as sulfathiazole (*Andersen, Friedberg, Vermehren & Vermehren*).

There is no statement as to whether while in the outpatient department the patient presented signs of hypersensitivity to alphasol, but on admission to the ear department exanthema developed so soon after administration of sulfathiazole that the reaction can hardly be taken as a primary reaction of sulfathiazole, but rather as a reaction of hypersensitivity on account of a previous sensitization.

On admission to the medical department sulfathiazole (chemosept) was again given on account of the elevated temperature, and in connection with this the temperature rose to 40.4° , and exanthema developed again, besides which the renal symptoms now manifested themselves by pains, albuminuria, hæmaturia, oliguria and elevated blood-urea. At the same time as the renal symptoms began the abdominal attacks also set in.

The whole of this symptom complex may also be taken as a response to a renewed administration of antigen. Further, the impression is conveyed that the high reaction on the part of the temperature in connection with the administration of sulfathiazole does not, as it were, fit into the whole tendency of the temperature curve, and also this rise may be read as an element in the reaction due to hypersensitivity. The same features are reflected, although to a smaller extent, in the rise of temperature following the first administration of sulfathiazole.

It is striking that the temperature drops very promptly, and that the exanthema quickly subsides despite the continued administration of sulfonamide (which has been changed to — as it is supposed to be — sensitizing alphasol). This might give rise to some hesitation in accepting the sulfathiazole as the exciting cause, but as the symptoms in periarteritis nodosa may be highly transient, this objection is hardly of decisive importance. It therefore seems reasonable to assume that the patient's disease has been caused by the sulfathiazole, in the way that he has become sensitized during treatment in the out-patient department, whereupon the vascular processes have been started at the first, or perhaps the second sulfathiazole medication in the medical department.

There may perhaps be reason to consider yet a possibility, viz. that the condition has developed within the period indicated, but has been provoked either by the allergen underlying the sinusitis and the asthma, or by some other allergen. The question then presents itself in which manner the sulfonamides may have caused an aggravation of the condition, as the coincidence between the second administration of sulfonamide and the development of the renal symptoms can hardly have been accidental, and the aggravation was indeed taken by the clinicians as an injury caused by sulfonamides.

Such an aggravation or complication of the lesion may be imagined

to have developed as a result of an occlusion of the renal tubules by crystals of sulfathiazole or by a so-called »distal tubular nephrosis« with subsequent hematuria and oliguria. However, it seems that affections of this type have not been present, as the course of the lesion is suggestive of a glomerular injury. Further, there is hardly any basis for assuming that, even if present processes of the kind here dealt with would exercise any accelerating effect on those vascular processes in the kidneys which were due to a possible basic condition, and in any case not on the vascular processes in the other organs, which now also began to develop rapidly. As a last possibility of an aggravation caused by the sulfathiazole there is an injury of an allergic nature, caused by sulfonamide, of the type mentioned in the introduction, which in turn is dependent upon a sensitization to the substance.

This last possibility thus takes us back to the assumption of a sulfonamide allergy and it will then be the simplest attitude to look at the whole picture from this point of view.

Summary.

A case is reported of periarteritis nodosa in a 20-year-old man who suffered from sinusitis and asthma. Throughout six months he was treated in the out-patient department with puncture and application to the maxillary sinuses of alphasol. As the condition did not improve operation was performed, and the patient was given sulfathiazole, after which an exanthema developed. On account of a rise in the temperature sulfathiazole was again given a fortnight later, after which exanthema developed once more and at the same time signs appeared suggesting injury to the kidneys with hematuria, oliguria and rise of the blood urea. In addition hereto, there also came clinical symptoms of periarteritis nodosa. The condition terminated fatally in the course of 12 weeks. Autopsy and microscopic examination disclosed typical periarteritis nodosa changes in practically all the organs. The processes were quite preponderantly fresh and must all of them be taken to have developed within the time the patient was staying in the hospital. The authors consider it most reasonable to assume that the condition was caused by sulfathiazole.

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ENDOPHLEBITIS HEPATICA OBLITERANS

By Gunnar Teilum.

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Numerous cases of obliterating vascular disorders of visceral localization have been described showing more or less characteristic pathologic-anatomical and clinical pictures. Of thromboangiitis obliterans Buerger, well known in its peripheral form, a few cases submitted to post-mortem examination have thus been described showing, in addition to typical findings in the arteries of the extremities, typical changes in various visceral vascular areas, e. g. in the coronary arteries (in connection with localized myofibrosis) and in the arteries of the gastro-intestinal tract (see Teilum, 1941). Moreover, there have been established purely central forms, localized to the brain or the lungs (Hadorn *et al.*), but still it has not been possible to draw any definite limit between the pulmonal form of Buerger's disease and arteriopathia pulmonalis (Ayerza), which, like Buerger's disease, has been perceived as an allergic vascular disorder and even in some cases a being of a genuine rheumatic nature because of its morphological resemblance to other rheumatic vascular disorders and its occurrence in connection with unquestionable cases of rheumatic disease of the heart (Bredt, Rössle). Cases of universal vasculitis in the fresh stage are also known, showing thromboangiitic changes in visceral vessels (with numerous infarcts, e. g. in the kidneys and the spleen), also in the aorta and in the arteries of the extremities. Arterial changes of this nature are generally considered to be thromboangiitis obliterans, whilst a similar disorder of the veins is termed thrombophlebitis migrans. The latter, too, is known to occur both in visceral and peripheral forms, the latter again being often associated with the peripheral form of Buerger's disease as disseminated phlebitis. It is, however, doubtful whether the so-called thrombophlebitis migrans constitutes a pathogenetic entity.

When Buerger's disease is localized to the small vessels, the changes often display the same histological picture as in *periarteritis nodosa*, which must be considered an allergic vascular disorder, closely related pathogenetically to *lupus erythematosus disseminatus* in which miliary allergic granulomata and necroses have been demonstrated in the serosa in addition to a number of other characteristic lesions of an allergic nature (Teilum, 1945—46).

The present publication deals with a very rare and, consequently, rather unknown obliterating vascular disorder occupying an exceptional position because of its strict localization to a single organ, the liver, which displays a more or less marked occlusion of the large and small hepatic veins. Already in 1846 thrombotic occlusion of the hepatic veins was observed by Budd, whilst only in 1899 Chiari described an *endophlebitis hepatica obliterans* as a disease *sui generis*, considering — in contrast with previous authors — an independent productive inflammation of the intima, i. e. an isolated endophlebitis without any changes of the circumference, as the essential. Like O. Meyer, Beitzke and others later on, Chiari supposed that it was a syphilitic vascular affection, a view that has proved to be untenable. As already mentioned, *endophlebitis hepatica obliterans* is of rare occurrence. In 1932 Inthorn gave a survey of the 54 cases described up to that year; later, the number has increased to 74 (König, 1944), whilst Kahn & Spring (1940) stated the number of cases reported to be about 60.

On closer examination considerable difference in the pathological-anatomical picture are seen. In some of the cases there is thus an occlusion of the venous openings into the vena cava inferior, in others special changes of the smaller veins are described. A histological description is lacking in the earlier cases, in the more recent ones descriptions are often found of thickened vascular walls, in particular of the intima, which is the only actual common feature. In some cases the authors have reckoned with a primary thrombosis with secondary organization. On the whole, it is probable that not all such cases of apparently primary occlusion of hepatic veins form an etiologic and pathogenetic entity. Especially in cases with a purely fibrous vascular obliteration without any histological characteristics a more thorough elucidation of the nature of the disease will be impossible. The clinical picture appearing in case of occlusion of the hepatic veins, and termed Chiari's syndrome, is also seen in secondary thrombosis, several cases of which have been described in polycythemia vera (*erythremia*) in which thrombotic processes on the whole play an important part (Oppenheimer, Cole, Sohval). Kelsey & Comfort, who, in 1945, described 20 cases of occlusion of hepatic veins, found this to be a chance finding in 16 cases, whilst it gave rise to symptoms in 4 cases only; apart from 1 case the affection was secondary in all the cases.

As to the nature of endophlebitis, different opinions have been

advanced in the course of time; it has, for instance, been considered a non-inflammatory degenerative sclerosis caused by chronic stasis, disturbances of circulation or thrombosis. On the basis of the case examined by Inthorn and Rössle the latter has stated the reasons for regarding endophlebitis hepatica obliterans as a special form of *allergic* inflammatory vascular disease in Klinge's sense of the term, a view that is endorsed by Coronini & Oberson and by Burkhardt. The etiology of the disease is obscure, but bacteriotoxic causes must be considered. In infants, too, (Wurm, Ewers), and in the newborn, cases of endophlebitis hepatica obliterans have been described. In Wurm's cases of bottle-fed infants a serous inflammation of the walls of the small veins was found, which Wurm ascribed to an alimentary intoxication, whilst in his case in a newborn child König reckoned with an intoxication from the mother. Endophlebitis hepatica obliterans in adults occurs especially at ages from 20 to 50 years, and is more frequent in females than in males. Most frequently the disease develops gradually in the course of a few months. In conformity with the progressive character of the pathologic-anatomical changes, the course has been fatal in all cases. The initial symptoms are less than characteristic. In some cases initial diarrhoeas occur, which have been associated partly with an alimentary-toxic etiology, partly with the stasis of the portal vein. Gradually, the main symptom becomes an increasing ascites with dehydration of the patient's organism (oliguria and polycythemia spuria). The diagnosis has hardly been established *intra vitam*, which to some extent is due to the fact that the disease is so rare and little known.

The following is a pathologic-anatomical description of a case of endophlebitis hepatica obliterans in which it was possible to follow the different stages of development of the disease in the large and small hepatic veins.

Case record: — The patient was a woman, aged 36 years, admitted to Department A of the University Hospital from Nov. 14th to Nov. 23rd 1911. Never rheumatic fever, scarlatina, nor frequent attacks of acute tonsillitis.

Three months ago she got febrile attacks with diarrhoea for 3 days. Since 3 weeks before admission, increasing abdominal distension, nausea and oliguria. No jaundice. Stools natural. No hematemesis.

Objective examination: Slight yellow discoloration of the sclerae but not of the skin. Neck and thorax: Nothing abnormal.

The abdomen: Distended, tense with marked ascites. Girth of abdomen: 92.5 cm. By *laparocentesis* clear ascites fluid (specific gravity 1004) was drawn out repeatedly, after which the liver was felt with a firm border two fingers' breadth below the costal margin. The *spleen* cannot be palpated.

Ascites fluid: No growth, no bacteria, no tubercle bacilli on cultivation.

Five days after the puncture, the ascites had recurred, but venous stasis was now observed (cyanosis and oedema) of the *left* upper extremity with increased venous pressure. Death occurred 4 days later, on Nov. 23rd.

Laboratory examinations: Icterus index: 19—15, prothrombin time: 54 seconds, urine: — (alb. sugar), hemoglobin: 110—116, Wassermann's test nega-



Fig. 1.

The vena cava inferior cut open, showing the obliterated hepatic veins.



Fig. 2.

Cut surface of the liver, showing obliterated hepatic veins and very marked signs of stasis.

tive. Blood-pressure 120/75, sedimentation rate: 1 mm., erythrocytes: 6.15 mill., index 0.87, leukocytes 9,800, serum protein 7.4 per cent.

Temperature 36.4° to 38.4° C.

Post-mortem examination showed fresh thrombosing of the axillary vein, the subclavian vein and of the lower part of the internal jugular vein of the left side.

The lungs: Hypostasis and marginal atelectases.

The pleura, pericardium and the heart: Nothing abnormal.

The peritoneum: 800 cc. yellowish ascites fluid. Marked stasis of the gastro-intestinal canal. Congestion of the veins of the stomach and of the lower part of the oesophagus.

The v. cava inferior: No thrombosis of the venous trunk proper, but where

the hepatic veins open out, obliteration was observed with not quite fresh adherent, partly organized thrombi protruding into the vena cava (Figure 1).

The veins of the lower extremities displayed no thrombosing.

The liver measured $21 \times 18 \times 10$ cm. and was of a dark bluish-red colour. There was diffuse whitish thickening of the capsule and slight shrivelling along the lower margin. No granulation, but diffus retracted areas on the lower anterior surface. A very marked stasis was observed, with very distinct nutmeg markings on the cut surface. The consistence was increased a good deal.

On the cut surface the large branches of the hepatic veins were seen to be the seat of almost complete occlusion with marked padding of the walls in



Fig. 3.

Hepatic vein with endophlebitic padding containing collagenic and elastic threads. v. Gieson-Hansen. $\times 8$.



Fig. 4.

Early endophlebitic phase. Solution, and proliferation of endothelium, fibrinous padding lining the wall. Hematoxylin-Eosin. $\times 160$.

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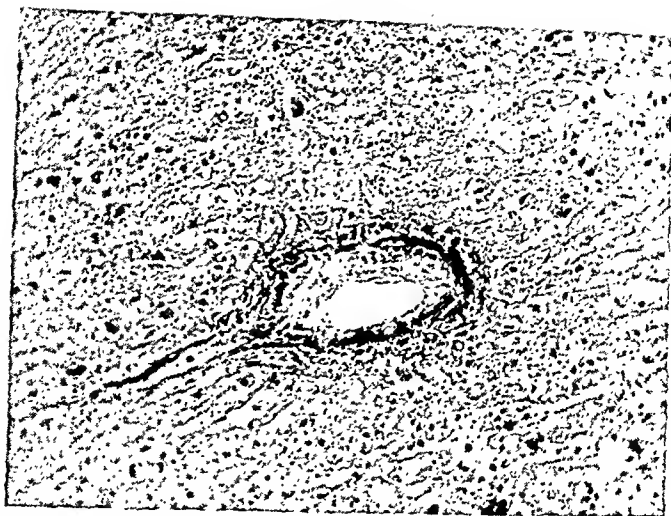


Fig. 5.

Branch of hepatic vein with cobwebby reticular structure with proliferated endothelium, blood corpuscles and thrombocytes. v. Gieson-Hansen, $\times 170$.



Fig. 6.

Venous polypus covered with endothelium, v. Gieson-Hansen, $\times 210$.

connection with thrombus formation (Figure 2). Downwards, towards the porta hepatis a firm whitish yellow infarct, measuring 5×4 cm., was found. The portal vein was the seat of secondary stagnation thrombus in the main branches, but displayed no padding.

The spleen measured $17 \times 10 \times 7$ cm., its weight being 550 gms. The surface was smooth, bluish red, and the capsule tight. No infarcts, the consistency firm.

The pancreas displayed diffuse small fatty necroses on the surface.

After the macroscopical findings there was no doubt that the case was one of endophlebitis hepatica obliterans, which diagnosis was corroborated by the histological examination.

The histological examination showed a very marked stasis of the liver

in addition to fresh as well as more long-standing endophlebitic occlusions of the hepatic veins. The changes had progressed most in the hepatic veins nearest to the openings into the vena cava, whilst more recent changes were found in the smallest branches, so that it was possible to follow the different stages of the development of the changes.

The large branches of the hepatic vein were found to be obliterated in part by a thick padding of organized masses, arranged in layers, interwoven by collagenic and elastic threads and containing larger and smaller blood-filled cavities (Figure 3). Whilst this padding lined the entire circumference of the vessel, the smaller branches of the hepatic vein displayed early stages

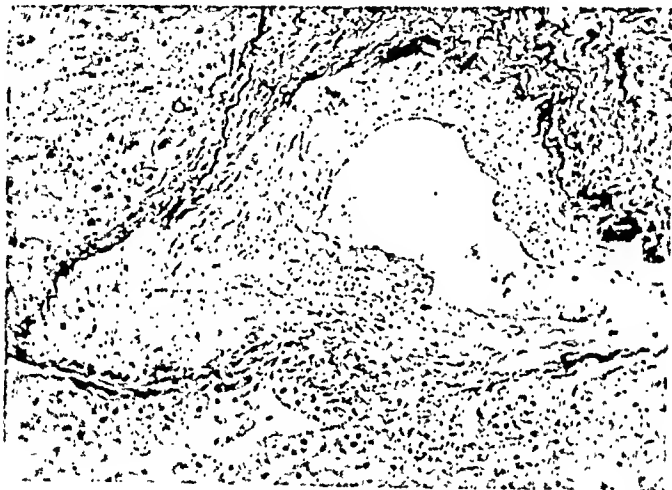


Fig. 7.

Hepatic vein with endophlebitic reticular structure. (Proliferation of the endothelium with finely granulated precipitation and incipient fibrosis).
v. Gieson-Hansen. $\times 170$.

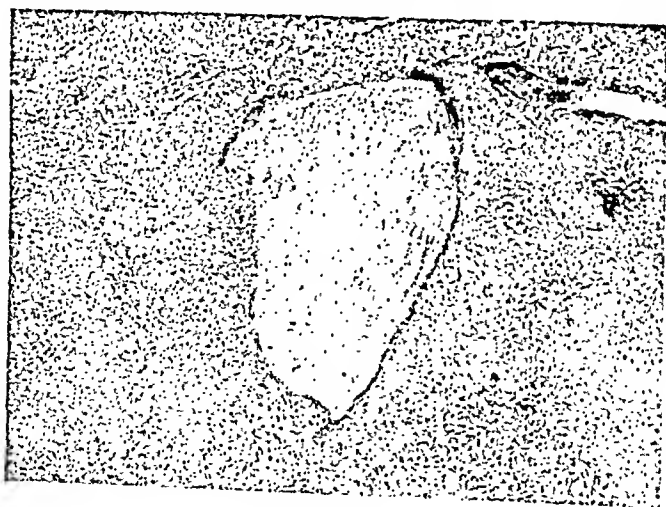


Fig. 8.

Hepatic vein with complete fibrous obliteration. v. Gieson-Hansen. $\times 34$.

with solution and proliferation of the endothelium, the walls being lined with fibrinous pads (Figure 4), also cobwebby reticular structures of proliferated endothelium with finely granulated precipitation of erythrocytes and blood platelets (Figure 5). Venous polypi covered with endothelium, the development of which has been explained by Rössle as a fibrinoid precipitation in the loosened proliferating endothelium, were found in a few of the smaller veins (Figure 6).

From such histological pictures transitions were found via endophlebitic reticular structures with incipient development of connective tissue (Figure 7) to complete fibrous obliterations (Figure 8).

The portal vein displayed no endophlebitic changes. In the splenic vein there were, in parts, faintly marked purely thrombotic processes, which must be regarded as secondary processes. The jugular and axillary veins showed fresh thrombotic changes without any signs of endophlebitis.

Epicrisis.

A case of typical endophlebitis hepatica obliterans is reported in a woman, aged 36, with rapidly increasing ascites. On post-mortem examination a very marked hepatic stasis was ascertained with almost complete occlusion of the hepatic veins, displaying marked padding of the walls in connection with secondary thrombosis. The histological examination showed solution and proliferation of the endothelium, with the formation of pads, venous polypi, and secondary thrombosing and fibrous obliterations, so that it was possible to follow the different stages of the changes.

Discussion.

This is a *primary* disorder, isolated to the hepatic veins with a characteristic pathologic-anatomical picture. Special mention may be made of the localizations both to the large and the small branches of the hepatic veins; of the well-defined delimitation of the process at the opening of the hepatic veins into the vena cava inferior (Figure 1), and of the fact that the changes are far more advanced in the large branches than in the small ones, which, however, displayed incipient solution and proliferation of the endothelium almost everywhere. Moreover, the changes are primarily of an endophlebitic nature, whilst the thrombosing is a purely secondary one. Similar changes could not be demonstrated in other organs or vascular areas.

The simultaneous occurrence of both fresh and more advanced changes affords the opportunity, otherwise only present in the case described by Inthorn and later by Rössle, of following the development of the changes and, at any rate in these cases, shows the same morphogenesis, whereas this is indefinable in most other cases with fibrous obliteration.

Inthorn and Rössle's case was that of a woman, aged 36, who for some months had suffered from radiating abdominal pain and, later, from rapidly increasing ascites. The patient died after a Talma's operation performed owing to suspicion of hypertrophic cirrhosis. Not

only the clinical but also the pathologic-anatomical picture showed a close conformity with what has been described above, with padding of the vascular walls, solution and proliferation of the endothelium with venous polypi (Figure 6).

Rössle stresses the points of resemblance to the changes in cases of allergic vasculitis, pointing out in particular the resemblance to the findings in the so-called primary sclerosis of the pulmonal artery previously reported by Bredt, and is inclined to consider the disorder a rheumatic (or rheumatoid) vascular disorder owing to the conformity with rheumatic vascular changes and corresponding experimental findings.

Neither in my case nor in that of Rössle the case history or the post-mortem findings did otherwise give any evidence at all of the presence of an allergic or a rheumatoid general affection (cf., for example, the allergic lesions in lupus erythematosus disseminatus and allied conditions described by the writer). Even though the possibility can hardly be denied that allergy plays a rôle in endophlebitis hepatica obliterans, it is, however, reasonable to emphasize the complete absence of allergic tissue lesions and vascular lesions in other organs and vascular areas; and the venous lesions in endophlebitis hepatica obliterans cannot be identified either with the venous changes of an unquestionably allergic nature which are seen in rheumatic and pararheumatic disorders, such as, for example, lupus erythematosus disseminatus.

Allergic lesions confined to a single organ or vascular area, are, however, by no means unknown, and the consequences of an intoxication from the intestinal canal, as supposed by the authors mentioned in the introduction, does not seem to be improbable.

The occurrence of initial diarrhoeas, as in the case reported here, might perhaps point in that direction.

The peripheral thrombosis in the veins of the upper extremity, in the subclavian and jugular veins has been regarded by me as having been caused by changes of the blood, resulting from the almost complete exclusion of the liver in connection with the dehydration (with secondary polycythemia), and not as a lesion that is parallel to the changes of the hepatic veins. Traumatization of the walls of the veins of the upper extremity when drawing blood samples and measuring the venous blood-pressure may possibly also have been of some localizing importance.

There can thus be no doubt that the present case and the one described by Inthorn and later by Rössle form a nosological entity, whereas in other cases of Chiari's syndrome we may be confronted with disorders of another etiology or pathogenesis. König's fetal form and the cases occurring in infants take up a special position, whilst other cases of occlusion of the hepatic veins constitute a mixed group, including cases of secondary thrombophlebitis and pure thromboses in polycythemia vera.

Summary.

A pathologic-anatomical description is given of a case of endophlebitis hepatica obliterans in a woman, aged 36 years. The clinical picture (Chiari's syndrome) had developed in the course of a few weeks with increasing ascites.

Post-mortem examination showed a very marked stasis of the liver with almost complete occlusion of the large branches of the hepatic veins. The histological examination revealed solution and proliferation of the endothelium in the small hepatic veins, with padding of the walls and venous polypi, secondary thrombosing and fibrous obliterations, so that it was possible to follow the different stages of development.

The case reported here and the case of endophlebitis hepatica obliterans described by Rössle (1933) can be said with certainty to constitute a nosological entity, whereas Chiari's syndrome generally represents a mixed group, including secondary thrombophlebitis and thrombosis of the hepatic veins, e. g. in polycythemia vera.

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STUDIES ON BACTERIA OF THE BALLERUP GROUP

By *Niels Harhoff*.

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In 1940 *Kauffmann & Møller*¹ described a bacterium, which — on account of its serological and cultural aspects — they referred to the *Salmonella* group under the designation of *S. ballerup*.

Subsequently several strains have been described that were near-related to this one (*Edwards*², *Monaci & Poli*³). As some of these strains differ culturally from the *Salmonella* group, all the strains belonging hereto have been entered into a separate and special bacterial group designated as the *Ballerup* group, even though it is near-related to the *Salmonella* group (*Kauffmann*⁴).

In The State Serum Institute, in recent years, several strains have been isolated that were found to belong to this group.

In a preceding paper⁵ a preliminary account has been given of 5 of these strains. Since, 10 additional strains of this group have been isolated, most of them in 1945. Thus altogether 16 strains of this type were isolated up to spring 1946, including the original *Ballerup* strain.

These strains have now been submitted to a more thorough investigation than in the preceding paper. For the sake of comparison various other strains have kindly been placed at my disposal by Dr. *Kauffmann*, namely the following strains:

Hormaeche orig.
Hormaeche 40.
Mustakallio 3205.
Mustakallio 4924.
Giovanardi 59.
Giovanardi 527.
L. C. 54.
Exeter 153.

These strains were examined culturally and serologically with regard to O and H antigens.

For certain reasons it has been impracticable to carry through this work completely, but it still seems appropriate and of some interest here to report the results obtained so far.

O Antigen.

The strains were examined for O antigen of various types as described by *Kauffmann*⁶.

The agglutination tests were carried out in dilutions 25 — 50 — 100 6400, and the results were read after incubation at 50° for 20 hours. These experiments were carried out with employment of pure O sera when such was available, otherwise with O-H sera. Both for the agglutination tests and for the absorption experiments the cultures have been boiled, so that the possibility of H reactions may be excluded.

In the preceding paper it had been demonstrated that 3 different O antigens were then found in the group, so that 3 different O types (see Table 1) could be set up as having the following antigenic formulae:

Original Ballerup strain:	XXIX ₁ , XXIX ₂ , XXIX ₃ .
2508/42	XXIX ₁ , XXIX ₂ .
8665/42	XXIX ₁ , XXIX ₃ .

Factor sera can be produced as follows:

Serum 2508/42 absorbed with strain 8665/42 gives XXIX ₂ ,
» 8665/42 » » » 2508/42 » XXIX ₃ .

Now agglutination tests were made with all the strains in sera produced by immunization with the 3 above-mentioned strains as representatives of the 3 types. Further, these 3 strains were submitted to agglutination tests in all the present O (or O-H) sera, in which sera also the homologous titer was determined. In this way, in nearly every instance, a positive reaction was obtained at a high titer (1600—6400), only 4 reactions showing a titer of 800, and 1 with a titer of 400 (Mustakallio 4924 in serum 8665/42), and it is not to be expected that the reactions lacking here would deviate essentially from the values recorded.

For determination of the O type all the strains were submitted to agglutination in the above-mentioned factor sera, and thus the strains were found to be referable to one of the types as follows:

3 strains were found to possess both factor XXIX₂ and factor XXIX₃, namely: the original Ballerup strain, and the strains 12727/42 and 16448/45. Both latter strains are able in absorption experiments completely to empty a Ballerup serum, and the original Ballerup is able to empty serum 16448/45. So undoubtedly the last strain is identical with the original Ballerup strain, and the same presumably applies also to strain 12727/42, but no serum was produced with this strain.

Table 1.
O Absorption.

100° culture		Serum S. ballerup absorbed with			
		1938/39	2508/42	8665/42	2508/42 and 8665/42
S. ballerup	XXIX ₁ —XXIX ₂ —XXIX ₃	200	200	100	0
1938/39	XXIX ₁ —XXIX ₂	0	0	200	
2508/42	XXIX ₁ —XXIX ₂	0	0	200	0
8665/42	XXIX ₁ —XXIX ₃	200	200	0	0

		Serum 1938/39 absorbed with		
		S. ballerup	2508/42	8665/42
S. ballerup	XXIX ₁ —XXIX ₂ —XXIX ₃	0	0	50
1938/39	XXIX ₁ —XXIX ₂	0	0	100
2508/42	XXIX ₁ —XXIX ₂	0	0	50
8665/42	XXIX ₁ —XXIX ₃	0	0	0

		Serum 2508/42 absorbed with		
		S. ballerup	1938/39	8665/42
S. ballerup	XXIX ₁ —XXIX ₂ —XXIX ₃	0	0	1600
1938/39	XXIX ₁ —XXIX ₂	0	0	1600
2508/42	XXIX ₁ —XXIX ₂	0	0	1600
8665/42	XXIX ₁ —XXIX ₃	0	0	0

		Serum 8665/42 absorbed with		
		S. ballerup	1938/39	2508/42
S. ballerup	XXIX ₁ —XXIX ₂ —XXIX ₃	0	400	400
1938/39	XXIX ₁ —XXIX ₂	0	0	0
2508/42	XXIX ₁ —XXIX ₂	0	0	0
8665/42	XXIX ₁ —XXIX ₃	0	400	400

Altogether 11 strains were found to possess the O antigens XXIX₁ and XXIX₂, namely, besides 2508/42, the following strains:

Hormacehe 40, L. C. 54, Exeter 153, 1938/39, 1939/39, 6699/42, 10527/45, 11204/45, 12457/45, 15339/45 and 28990/45. All these strains were able completely to empty serum 2508/42, and the latter was able to empty immune sera produced with the first 6 of the strains mentioned, while no immune serum was produced with the last 5 strains.

The last 9 strains possess antigens XXIX₁ and XXIX₂. Immune sera were produced with 8 of these strains, and all these sera were emptied completely by strain 8665/42. Besides, strains 10192/45, 20627/45 and Mustakallio 3205 were able completely to empty serum 8665/42, whereas the remaining strains either gave varying results or failed completely to empty serum 8665/42. These experiments indicate that type XXIX₃ possesses some partial antigen, and that possibly a change in form is taking place too.

Furthermore, the Danish strains were also examined for the pre-

sence of the Vi antigen by means of slide agglutination tests on several colonies from each culture: besides, some of the strains were examined also by cultivation on ascites plates. Only 2 strains showed the presence of this antigen, namely: the original Ballerup strain and strain 12727/42. Both showed distinctly a change in form, both macroscopically in the type of growth and serologically in the slide agglutination tests.

H Antigen.

Altogether 9 of the strains were non-motile in broth culture as well as in semifluid agar. namely: strains 1938/39, 1939/39, 6699/42, 10527/45, 11204/45, 12457/45, 15339/45 and 28990/45 — all of the O type XXIX₁ and XXIX₂.

All the remaining strains were lively motile. H sera were produced against these strains, excepting 12727/42 and 10192/45. Agglutination experiments were carried out by examination of all the strains in all the sera (see Table 2). Besides, tests were also made to see whether these strains would agglutinate in the available Salmonella H sera, and no positive reaction was found in any instance.

The results here obtained are not clear-cut, requiring a more thorough analysis, with simultaneous tests for any possible change in form. Still, the results show something.

The original Ballerup strain presented no overlapping with the remaining strains. 1 strain (12727/42), with which no immune serum had been produced, was agglutinated to the titer limit in serum produced with the Ballerup strain, and it also was able to empty this serum; in addition, however, it gave a couple of weaker reactions in other sera. Here it is to be mentioned that this strain was also the only one in possession of the Vi antigen, and hence it probably belongs to the original Ballerup type. The H antigen of the other XXIX₁, XXIX₂, XXIX₃ strain showed no overlapping.

The only motile strain possessing antigens XXIX₁ and XXIX₂ that has been found here in the State Serum Institute showed no antigenic fellowship with other strains, whereas the 3 following strains presented closely related antigens, perhaps identical, but no absorption experiments to this effect were performed.

Within the XXIX₁, XXIX₃ strains 4 strains were found to be near-related, agglutinating to the titer limit — or at any rate at a high titer — in their mutual sera. Still, absorption experiments proved them to be different (Table 3). No complete analysis, including a possible change of form, has been carried out. Strain 8665/42 appears to be an independent type, although it gives some overlapping to the above-mentioned strains. Strain 10192/45 is agglutinated to the titer limit by

Table 2.

H Agglutination.

Culture	Serum									
Ballerup orig.	6400	6400	1600	+	?	3200				
12727/42										
16448/45		3200								
2508/42			6400							
Hormaeche 40			6400	1600	3200					
L. C. 54			400	6400	6400					
Exeter 153			400		1600					
8665/42						6400				
10192/45						6400				
14433/45							6400	800	6400	400?
20627/45							6400	6400	6400	400?
Mustakallio 3205							6400	400	6400	800?
Giovanardi 527							6400	400	6400	200?
Giovanardi 59									6400	
Hormaeche orig.										6400

1600?

? = the reactions have varied.

serum 8665/42, but no serum has been produced with this strain and thus its aspects have not been established. Also Giovanardi 59 and the original Hormaeche appear to be types by themselves — although with some overlapping.

Table 3.
H Absorption.

Culture	14433/45 unabsorbed	Serum	
		14433/45 absorbed with 3205	527
14433/45	6400	0	≥ 25
Mustakallio 3205	6400	0	0
Giovanardi 527	6400		0
	20627/45 unabsorbed	20627/45 absorbed with	
		14433/45	3205
14433/45	800	0	0
20627/45	6400	≡ 800	≡ 800
Mustakallio 3205	400	≡ 25	0
Giovanardi 527	400	0	0
	3205 unabsorbed	3205 absorbed with	
		14433/45	527
14433/45	6400	0	200
20627/45	6400		≡ 200
Mustakallio 3205	6400	0	100
Giovanardi 527	6400		0
	527 unabsorbed	527 absorbed with	
		14433/45	3205
14433/45	6400	0	0
20627/45	6400	0	0
Mustakallio 3205	6400	25	0
Giovanardi 527	6400	400	400

Cultural Examinations.

All the strains were examined culturally by growing on a number of media. Thus they all were found to ferment mannite, sorbite, arabinose, xylose, rhamnose, maltose and trehalose within 24 hours, whereas they failed to ferment adonite, inosite, l- and i-tartrate, not even in 30 days. The other reactions are evident from Table 4.

Table 4.
Cultural reactions.

Strain	Motility	Lactose	Saccharose	Salicine	Dulcitol	d-Tartrate	Citrate	Stern	H ₂ S	Ammonium-glucose	Ammonium-citrate	Mucate	Brilliant green
Ballerup orig.	+	0	0		+1	+7-8	+2	+	+	+7	+5	+1	r ¹
12727/42	+	0	0	(+)+ ⁵	+1	+8	+8	+	+	+2	+2	+2	r ¹
16148/45	+	0	0	(+)+ ⁸	+1	+8	+8			+3	+1	+2	r ¹
2508/42	+	0	0	(+)+ ¹⁰	+1	+11	+1	+	+1	+1	+1	+	r
Hormaeche 40	+	0	0	0 ¹¹	+	+5	+1	+	+2	+2	+2	+1	r
L. C. 51	+	0	0	+2	+	+5	+1	+	+	+2	+1	+2	r
Exeter 153	+	0	0	+5	+	+6	+4-5	+	+1	+3	+3	+1	r
1938/39	—	0	0	+7	+1	+14	+5	+	+	+1	+1	+1	r
1939/39	—	0	0	+8	+1	+10	+5	+	+2	+1	+1	+1	r
6699/42	—	0	0	(+)+ ¹¹	+1	+11	+5	+	+1	+1	+1	+1	r
10527/45	—	0	0	(+)+ ⁸	+1	+8	+8			+2	+1	+2	r ¹
11201/45	—	0	0	+6	+1	+8	+8			+2	+1	+2	r ¹
12457/45	—	0	0	+8	+1	+8	+8			+2	+1	+2	r ¹
15339/45	—	0	0	(+)+ ⁸	+1	+14	+8			+2	+1	+1	r ¹
28990/45	—	0	0	(+)+ ⁸	+1	+8	+8			+2	+2	+2	r ¹
8665/42	+	(+)+ ⁵	0	(+)+ ¹¹	0	+6	+1	+	+2	+1	+1	+	r ¹
10192/45	+	+5-5	0	(+)+ ⁹	0	+8	+8			+2	+5	+2	gr ¹
14133/45	+	+2	0	(+)+ ⁸	0	+14	+8			+1	+1	+2	gr ¹
20627/45	+	0	0	+10	0	+11				+2	+2	+2	r
Mustakallio 3205	+	+1	0	(+)+ ¹⁰	+	+6	+2-4	+	+2	+2	+1	+	r
Giovanardi 527	+	+1	0	(+)+ ¹³	0	+6-8	+2	+	+	+2	+1	+1	gr ¹
Giovanardi 59	+	+1	0	+7	0	+7	+3-6	+	+	+3	+1	+1	gr ¹
Hormaeche orig.	+	+27	+10	+7	+	+5	+3	+	+2	+3	+10	+2	r
Mustakallio 4924	—	+10	0	+6	+	+6	+2-4	+	+4	+2	+1	+1	r

0 = no reaction within 30 days.

+2 = positive after 2 days.

no number: the day has not been recorded. By Stern's glycerin fuchsin broth
+ means that the medium was red on the 8th day.

r¹ = red after one day.

gr = green.

Empty spaces: the reaction has not been performed.

From Table 4 it will be noticed that most of the strains possessing antigens XXIX₁, XXIX₃ fermented lactose in 1—5 days, a few of them later, and 1 of them fermented saccharose late. The remaining strains did not ferment lactose and saccharose. On brilliant-green plates, as was to be expected, most of the strains grew as red colonies, some of the lactose-positive strains yielded red colonies, others green ones.

Of the XXIX₁, XXIX₃ strains 5 were dulcitate-negative, the remaining positive.

Conclusion.

By means of the O antigens it is practicable to classify the Ballerup group into 3 groups, possessing the following antigens: XXIX₁, XXIX₂ and XXIX₃; XXIX₁ and XXIX₂; and XXIX₁ and XXIX₃. Possibly the last group may be divided into subgroups.

8 strains of the XXIX₁, XXIX₂ group and 1 strain of the XXIX₁ and XXIX₃ group are non-motile. The motile strains possess H antigens showing complicated aspects that have not yet been settled.

Culturally this group reminds a good deal of the Salmonella group, but most of the XXIX₁, XXIX₃ strains ferment lactose, while one of them ferments saccharose too.

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BREEDING OF A COLONY OF WHITE MICE FREE OF ENCEPHALOMYELITIS VIRUS

By Herdis von Magnus and Preben von Magnus.

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Encephalomyelitis virus of mice, which was first isolated by Theiler (1) from the cords and brains in naturally occurring paralytic cases and later by Olitsky (2) from the intestines of normal stock mice, resembles human poliomyelitis virus in various ways: The clinical picture shows flaccid paralysis with practically no encephalitic symptoms. The virus is found in the intestines and excreted with the stools not only in paralyzed mice, but also in asymptomatic animals. The histological picture and the incidence of naturally occurring cases are also comparable with the findings in human poliomyelitis.

Strains of this virus are designated TO (Theiler Original) (3), to distinguish them from similar mouse viruses (FA, GD VII) later isolated by Theiler and Gard (4) and Melnick and Riordan (5). These latter viruses differ in essential qualities from the TO virus (3,5).

Normal colonies of laboratory mice investigated here and elsewhere have been found to harbor TO virus. The virus can be demonstrated in the intestines and intestinal contents from the age of three weeks and onwards for a period of several weeks.

It seemed likely that a comparative study of a normal »infected« mouse colony and a colony totally free from TO virus would offer a good opportunity for studying some of the problems concerning natural infection with and immunity to TO virus. So some years ago we attempted to breed a TO-free stock of mice.

We intended to avoid the »natural« infection of baby mice by using white rats to nurse them. White rats have never been found to harbor TO virus. The method used was as follows:

A number of pregnant female mice close to term were washed carefully with 96 % alcohol and placed separately in sterile boxes containing sterile sawdust. As soon as one of the mice had delivered, the babies were transferred to another sterile box. A white rat, which

had delivered the same day, was washed with alcohol and placed in the same cage. Although the rat ate the newborn mice in most cases, occasionally it adopted the mice and nursed them. During this period the rats were kept in a well cleaned room and fed sterilized food (bread and grain, tap water, and in some cases pasteurized milk). The animal caretakers changed their coats and washed their hands carefully before handling anything in the room.

After this one »passage through rats« the new colony was continued by mouse in-breeding, and the precautions to avoid contamination with TO were continued.

These procedures resulted in 1945 in a colony in which no TO virus could be recovered from the intestines of the mice at any age. But somehow the colony once became infected with TO virus. Another time the stock was discarded because of infection with bacillus piliformis (Tyzzers disease). It was not until the spring of 1948 that the supply from a new breed became sufficient enough to permit experiments for a period of 3 months with adequate numbers of mice. However, at the end of this period the colony has become infected with TO.

This latest colony of isolated mice (L-mice) was started in October 1947. It has been tested monthly from January 1948, and for 6 months no virus could be demonstrated in the intestines of mice, which were tested immediately after their removal from the isolation room.

The test for TO virus is carried out by inoculating a 20 % etherized suspension of intestines and intestinal contents from 5 mice (usually 6—8 weeks old) intracerebrally into 20 white mice, 4 weeks old.

The same procedure performed on intestines and contents from 5 normal mice (A-mice) 3—12 weeks old, results in paralysis in 10—18 out of 20 inoculated animals during an observation period of 28 days.

Normal adult mice are very little susceptible to peripheral inoculation with TO virus (1, 6, 7, 8), and this has been found true also with baby-mice (8). However, preliminary experiments have suggested that a difference in resistance exists in our two groups of mice, the virus-free mice (L-mice) and ordinary white mice (A-mice).

Subcutaneous and oral inoculation of baby mice produces about 100 % paralysis in L-mice, while A-mice are practically insusceptible. (However, a few litters from the A-mice are very susceptible to TO virus, as has been previously shown by Ørskov and Krag Andersen (8)).

Even the more vigorous challenge — intracerebral inoculation — shows a clear difference. TO virus titers from 10 to 100 times further in adult L-mice than in A-mice, the difference increasing with age.

A carrier state can be produced in L-mice without any accompanying paralysis by contaminating the cages with stools from normal mice.

Further details about the experiments will be published elsewhere.

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MORPHOLOGICAL STUDIES OF THE NORMAL
GROWTH OF A HUMAN TUBERCLE STRAIN, AND THE
EFFECTS OF SOME ANTIBACTERIAL SUBSTANCES
ON SAME*)

By *Einar Espersen.*

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Since Robert Koch demonstrated the tubercle bacillus in 1882 a number of workers have studied it, and it is fairly safe to say that hardly any other bacterium has been the subject of such different opinions.

In addition to the classical acid-fast rod there have been descriptions of non-acid-fast rods, as well as granular forms, fungoid forms and filterable forms. I shall mention merely some of the main points in the very comprehensive literature on the subject.

In 1907 Much²⁴⁾ described the granules that bear his name. In some cases of domestic animals (calves, sheep, pigs) with miliary tuberculosis, in the lungs of which he had been unable to find TB by means of Ziehl-Neelsen staining, by employing a modified Gram staining he demonstrated fine, Gram-positive granules, some singly, others in rows of up to five, and fine Gram-positive rods. After incubating pieces of lung from the animals in serum tubes he was able after three days by means of Gram-staining to show fine granules and fine rods, but nothing when he used Ziehl-Neelsen staining. After six or seven days, Gram-staining showed numerous rods, while Ziehl-Neelsen staining revealed acid-fast rods.

Inoculating pieces of lung into guinea-pigs produced widespread tuberculosis, and acid-fast TB were found in the organs of the animals.

Likewise, in pus from cold abscesses in man, in which he was unable to find TB with Ziehl-Neelsen staining, he found similar Gram-positive granules and rods. After infecting guinea pigs intraperitoneally with the pus he was able after 24 hours to demonstrate Gram-positive granules, after two-three days Gram-positive rods and fine, acid-fast rods by Ziehl-Neelsen staining. Killed after six weeks the guinea-pigs showed tuberculosis with acid-fast TB

*) Carried out with aid from Miss P. A. Brandt's Bequest.

in the organs. From this Much deduced that the Gram-stainable granules had developed into Gram-stainable rods which, after impregnation with fatty-acid substance, developed into acid-fast rods.

From his experiments Much concludes that there is a granular form of tubercle bacillus which does not stain by the Ziehl-Neelsen method, and that this granular form is virulent. It may be found as the only demonstrable form in the tuberculous organs or together with fine rods. There are transitions from the granular form to the fine, Gram-positive rods and on to the acid-fast rods and granules that are Ziehl-Neelsen positive.

In 1929 Morton Kahn¹⁸ published his theory of the life cycle of the tubercle bacillus. He examined a strain, H₃₇, in hanging micro-drops of Long's medium in moist chambers. He succeeded in isolating a single or a few tubercle bacilli in each drop. He drew the conclusion from his experiments that H₃₇ multiplies not only by simple division, but also by means of a more complicated reproduction: a) Initial segmentation of the rod into three or more ovoid elements. b) Division of these elements into diplococcoid forms. c) Followed by grouping and reduction of the elements into a crowd of dust-fine particles, from which small and fine rods shoot out. d) Later development of these fine rods into mature tubercle bacilli.

The coccoid and diplococcoid elements are acid-fast, whereas the very fine particles are non-acid-fast; the latter may possibly be the non-acid-fast granules described by Much.

In Denmark Kahn's work was critically examined in 1932 by Orskov¹⁹ in a brilliant work on the initial growth of the tubercle bacillus. Orskov repeated Kahn's experiments with the hanging-drop in a moist chamber, but arrived at the conclusion that the changes described by Kahn were not an expression of growth of the bacillus but due to its degeneration in conjunction with crystallizations from the medium.

Orskov also studied the initial growth of the tubercle bacillus by direct agar microscopy on sauton agar and pointed out a certain resemblance to the growth of ray-fungi, including angular growth and the frequent occurrence of true branching — in the human strain too — as signs of relationship between ray-fungi and mycobacteria.

In 1934 Wychoff²⁰ showed — by means of micro-film recordings — that in young cultures the tubercle bacillus grows in length before fission, whereas in old cultures fission takes place without previous prolongation, so that the bacteria become shorter and shorter during growth until coccoid forms appear.

A new work by Kahn & Nonidez²¹ appeared in 1936. On examining stained paraffin sections (Ziehl-Neelsen) from young growth pellicles on Long's fluid medium and colonies on Petroff's egg medium they found that the peripheral parts of the tubercle bacillus colony consists chiefly of non-acid-fast granules and coccoid forms, whereas the central parts of the colony are made up of chiefly acid-fast rods. They state that the granular and coccoid forms originate by segmentation of the rods under conditions where the rate of fission is greater than the ability of the microbe to grow in length. They consider them to be the youngest forms of the tubercle bacillus, as they are found in abundance in young colonies, but only few in old ones; as their number is highest in the periphery, they assume that growth is most rapid there.

The non-acid-fastness is explained by these authors by saying that in this phase the bacillus has a metabolism which does not permit of the development of the acid-fast property.

Vera & Rettger²², 1939, studied four tubercle bacillus strains in micro-culture on Long's agar in a moist chamber directly under a cover glass. Their conclusions were that the usual method of reproduction of the tubercle bacil-

lus is fission. Branching occurs occasionally. No other mechanism of reproduction was observed. The development of many variant forms of cells was easily induced by using media poor in nutriment and reducing the oxygen tension.

In 1944 Yegian & Porter⁴⁰ advanced their theory that many of the non-acid-fast forms are free artificial products due to traumatization. Young cells are more easily destroyed than old ones; the more pains are taken with the smearing, the more numerous are the non-acid-fast rods and granules, the destruction of the cells involving loss of acid-fastness.

Laporte²⁰, 1942, examined the autolysis of the tubercle bacillus and described the degenerative phenomena in old cultures as follows: First phase, conversion into granules of the bacillus, which still retains the classical rod form. Second phase, liberation of granules with the dissolution of ectoplasm. Third phase, loss of acid-fastness and progressive fragmentation of the free granules. As a rule the second and third phases proceed together. The first phase may be absent, or it may not be followed by the other two.

Granular fragmentation with loss of acid-fastness may be seen in cultures killed with toluol, chloroform, thymol and ether (not formalin or heat, which kill ferments). Oxygen is necessary for autolysis.

The origin of the tubercle bacillus from and transition to fungus have been described particularly by Swedish workers.

Reenstjerna²⁷, 1912, from the sputum of pulmonary tuberculosis patients isolated three forms of fungi of the oidium type, Gram-positive but not acid-fast. In pure cultures of these in glycerine broth 7 and 17 days old he found acid-fast rods like tubercle bacilli, which he assumed originated from the fungi. Inoculation into rabbits a month later, however, did not produce tuberculosis.

Conversely, after transplanting known human laboratory strains of Koch's bacillus in glycerine broth in its turbid phase from two to seven days he was able to demonstrate diphtheroid, non-acid-fast but Gram-positive rods, whereas the pellicle on the surface of the glycerine broth always consisted of acid-fast rods.

He also succeeded with four human tubercle bacillus strains in provoking the formation of non-acid-fast, Gram-positive, large «cocci» resembling yeast cells and very like the aforesaid fungus of the oidium type.

Gullberg⁹, 1938, in sputum from two pulmonary tuberculosis patients and in the blood of a patient with high fever and erythema nodosum found a non-acid-fast, but Gram-positive yeast-cell-like fungus (of oidium type). After preparing single-cell cultures and repeated transplantations in glycerine broth he was twice able in the course of two years to demonstrate the occurrence of acid-fast granules and rods of exactly the same appearance as Koch's bacillus. Inoculation into guinea-pigs produced in one animal a caseous inguinal gland, in the pus from which he found acid-fast rods. The animal died of cachexia after two months; a few acid-fast rods were found in the lungs. Several of the other guinea-pigs also died of cachexia, but with no sign of tuberculosis.

Hollström¹², 1942, continued Reenstjerna's work on non-acid-fast, diphtheroid rods. By means of a special arrangement he was able to withdraw samples from the bottom of a glycerine-broth culture of tubercle bacilli without touching the acid-fast TB on the surface. After eight days he was able to observe acid-fast granules in many of the diphtheroid rods and separately from them, the number increasing during the next four days. On the twelfth day, in among non-acid-fast and partly acid-fast elements he found granulated rods which were completely acid-fast. After two more days he also observed small, acid-fast corpuscles. On the twentieth day, especially after centrifuging, besides the above forms he also found a few fungus cells of yeast-cell

type, the majority containing acid-fast granules. Mixed infection being out of the question, and as these fungus cells were never found on the surface of the culture, he assumed that some of the diphtheroid rods growing in the oxygen-poor stratum at the bottom of the medium must have developed into fungus forms.

After preparing single-cell cultures of the non-acid-fast, diphtheroid rods and inoculating into guinea-pigs, he was able in two instances to demonstrate acid-fast rods in them, and in one of these animals typical tuberculous changes. The author assumed that the non-acid-fast diphtheroid rods developed in the animals into acid-fast rods.

I shall not here go into the question of the filterable tuberculosis virus, first described by Fontes⁵, 1910, later by Vaudremer³⁵, 1923, who was also able to cultivate this virus on suitable media, Calmette², 1928, and others.

More recent studies, Gloyne, Glover & Griffith⁸, 1929, Ørskov & K. A. Jensen⁴³, 1930, Vera & Rettger³⁶, 1939, and Soltys & Wilson³³, 1944, failed to verify the existence of a tuberculosis virus of this kind.

Own Investigations.

For these investigations I employed a well-known virulent human tubercle bacillus strain, Humanus E₅.

They were made on microcultures on solid medium in a moist chamber; the individual bacilli were observed direct under the cover-glass with an immersion lens and at suitable intervals.

The method has not previously been employed successfully in Denmark. The principle in the procedure is Ørskov's⁴² method for the pure cultivation of bacteria, amplified by K. A. Jensen¹⁴ for measuring bacterial growth. As I have made some changes in the method for the purpose of studying tubercle bacilli, and as painstaking technique is of great importance if a successful result is to be obtained, I shall describe it here.

Technique.

The moist chamber consists of a four-sided metal frame, 4 mm. high, 30 × 22 mm. in length and breadth. This ring is paraffined on the inner side and secured by means of paraffin to an ordinary slide. The ceiling of the chamber is a sterile cover-glass 24 × 32 mm. (fig. 1).

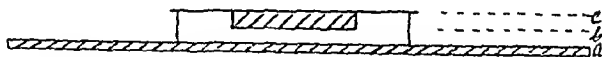


Fig. 1.

The moist chamber. a. Slide, b. metal ring, c. cover-glass and block of medium.

The cover-glass is wiped with non-greasy paper and sterilized by careful flaming. Hanging in the chamber on the underside of the cover-glass, with the inoculated surface against the glass, is a block of medium 2–3 mm. thick and about 1.5 × 2 cm. in breadth and length, a principle first employed by

Emil Chr. Hansen¹⁰, 1883, who followed the development of saccharomycetes from a single cell in gelatine culture in a moist chamber on the underside of a cover-glass directly under the microscope. Hill¹¹, 1902, later employed it for studying bacteria on agar.

The block of agar is fixed to the cover-glass along the ends with molten paraffin; this prevents the preparation from being moved and making it impossible to find the same cells again. By suspending the agar block under the cover-glass the air in the chamber, especially the oxygen, can diffuse freely up through the agar; as we have to do with the cultivation of an aerobic bacterium, this is important. A further advantage is that any slight difference in the thickness of the block as a result of an imperfectly plane Petri plate will be of no significance.

When the agar block is secured the cover-glass is placed over the chamber and sealed to it with molten paraffin. The slide is then placed under the microscope and fixed exactly by means of the clamps on the stage. Using the immersion lens, one finds a suitable field, and the bacteria in it are drawn on squared paper with the aid of an ocularmicrometer. The position of nonius is then marked. By means of this mark and the mutual positions of the bacteria, perhaps a characteristic group of cells, it is possible to recognize the same field. The individual bacterium may now be observed at suitable intervals for months if necessary.

The microscope used is a monocular Zeiss microscope No. 33040. Eye-piece marked: 10 X. The eye-piece is fitted with an ocularmicrometer. Immersion lens marked: H. I. ⁹⁰_{1.25-0.8}. The stage can be adjusted very accurately.

For illumination I used a powerful, but stopped-down light, 40-60 c.p., with which the bacteria can be seen more distinctly.

Medium.

The medium which I have found to be best for cultivating tubercle bacilli under a cover-glass is serum-tween-mannitol-sauton-agar, i.e. Sauton's medium with tween 80 0.05 %, serum albumin 0.5 %*), mannitol 0.2 % and agar 1.8 %.

Sauton's medium:	g.
Magnesium sulphate	2
Citric acid	8
Secondary potassium phosphate	2
Asparagine	16
Ferriammoniumcitrate	0.20
Glycerine	c. c. 240
Mains water	" 3800.

Neutralization with ammonical water to pH. 7.2.

Having found in the course of my first experiments that glycerine was lytic to the tubercle bacillus, I removed the glycerine from the Sauton medium.

Serum-tween-mannitol-sauton-agar is almost wholly clear, only faintly opalescent and therefore suitable.

*) The serum albumin was prepared by Dr. Hansen, chemist at the State Serum Institute. Originally I used human serum albumin, later bovine serum albumin.

It should just be mentioned that I also tried Dubos' medium with agar, but the cells on this are somewhat smaller and therefore more difficult to observe, while growth is somewhat slower, for which reasons I abandoned it.

With the technique employed I was also able to cultivate under cover-glass on sauton-agar and ordinary agar.

For inoculation I used cultures from Dubos' fluid medium, which gives rapid growth from minimal inocula in deep cultures.

The following was the composition of Dubos' medium:

	g.
KH ₂ PO ₄	1
Na ₂ HPO ₄ , 12 H ₂ O	6.25
Na ₂ citrate	1.50
MgSO ₄ , 7 H ₂ O	0.6
Asparagine, 20 c. c. of 10 % solution.	
Mannitol	2
Tween 80, 5 c. c. of 10 % solution.	
Ferriammoniumcitrate	0.1
Serum-albumin	2
Distilled water	to 1000 c. c.

The medium is adjusted to pH 7.0-7.2. The serum albumin is dissolved in distilled water, 5 % solution, neutralised and filtered twice through a Seitz filter. The serum albumin is added the medium aseptically after autoclaving. The medium is divided into tubes, 5 c. c. in each.

Inoculum.

The inoculum must be dense, e.g. one cell to each 12 μ^2 , and yet as far as possible it must consist of isolated cells or small groups of cells; a loopful of an 8-14 day culture in Dubos' medium will usually suffice. In some instances I employed a small loopful of a bacterial suspension.

The inoculum is placed on an aseptically cut, moderately dry agar block and spread over it as evenly as possible. Or one may first spread half of the block and then the other half, stroking from the first half over to the second; this gives a different inoculum density on the two halves.

Effectiveness of Method.

It is unnecessary to introduce water into the chamber; the medium in the chamber will keep for several months, six to nine, without drying up. Nor is a supply of air or oxygen necessary. As far as possible the microcultures should not be exposed to the light and should be taken from the thermostat for microscopy for short periods only.

The effectiveness of the method is shown by the fact that if a seven to fourteen-day culture from Dubos' medium is transplanted there will be a germination percentage of 95 to 100 as well as diffuse growth in the course of seven to fourteen days, if the inoculum has a density of about one cell per 12 μ^2 .

The cultures can remain alive for many months; I have seeded from cultures four, seven and nine months old and obtained growth in every case.

The method is suitable for studying the influence of various substances on the growth of the tubercle bacillus; one compares the growth in several moist chambers simultaneously; even very slight influences can be detected by this means.

Normal Growth of Tubercle Bacillus.

Immediately after seeding from Dubos' fluid medium in the growth phase, with cultures seven to fourteen days old, the bacilli will be seen in the form of non-motile, thin rods with slightly rounded ends and very faintly refractory to light. Closer examination will reveal various numbers, usually two to six, rather more refractile granules in them.

The bacilli are straight or slightly curved, some lying at angles to one another. The length varies from 2 to 4 μ (fig. 2). After 24 hours the cells are seen to have grown in length and thickness, some having become curved and entered upon the fission phase. A very few will have divided entirely. The cells are now more refractile and granules become more distinct. They are now 4 to 6 μ long (fig. 3).

In the second day fission is lively. Fission takes place transversally, the elongated or curved cell dividing across the middle. After fission the bacteria may lie in line, though often with slight parallel displacement, or they may form obtuse angles with one another. This angle is presumably a form of incomplete fission, the two cells being connected after fission by thin bridges of protoplasm.

As a rule these bridges are invisible, but in some cases when the cells have been very large, 8 to 10 μ , under the influence of various substances, the bridges were quite distinct.

As the one or both of the angle-forming bacteria move closer together the angle becomes more acute, until at last they lie parallel. This is a very characteristic mode of fission (fig. 4).

The new cells quickly divide again, and in the course of three or four days fission proceeds at increasing velocities, resulting in small colonies; in these colonies the cells may often be seen arranged in parallel streaks or swarms — again perhaps owing to the aforesaid protoplasm bridges.

During the rapid growth on the fifth and sixth days the cells decrease again in size to the original 2—4 μ , and granulation is less prominent.

In the course of the seventh to tenth day the growth will usually be diffuse in the slide.

If a thin inoculum has been used, whereby growth does not become

diffuse, it will be seen that in the colonies the velocity of growth after reaching a maximum falls again until growth almost ceases in the course of about three weeks.

How does growth proceed in the colonies? It is easy to see that there is peripheral growth, for there we can follow the individual cells; division proceeds in the usual manner by transversal fission of the elongated and perhaps curved rods and not, as Kahn & Nonidez¹⁰) assumed, via a granule phase.

Within the colony it is impossible to follow a single cell. Nevertheless it is clear that growth is proceeding, because the peripheral cells can be seen to be pushed farther out without dividing, while at the same time the number of cells in the colony increases.

Branching.

In the human tubercle bacillus strain with which I have worked there was extremely little genuine branching; I observed it definitely only a few times, once after transplanting a fourteen-day culture, after transplanting a six-weeks culture and after transplanting a four-months culture (in the latter instance two examples of Y-branching), and finally, after transplanting a 16-days culture under conditions where access of air to the culture was prevented.

Branching after transplanting old cultures is not peculiar to the tubercle bacillus. Gardner⁷), 1925, found branched or Y-forms (one Y-form for every 2000 simple elements) after seeding two to ten months' cultures of bac. coli, bac. dysent. (Shiga), bac. dysent. (Flexner), bac. typhosus, vibr. chol., bac. paratyphos, B and C.

Miche²³), 1909, who studied a human strain by the hanging drop method, states that branching undoubtedly occurs, but very rarely. On the other hand, Ørskov⁴¹) considers that branching occurs as a normal form of division in the human type too. According to Vera & Rettger³⁰), branching does occur occasionally, but the usual form of division is by transversal fission.

Granules.

In every culture of tubercle bacilli one finds a varying number of granules in addition to the rod-shaped bacilli. In size they correspond almost to the thickness of the bacilli (0.3 to 0.5 μ). In the foregoing I said that the rod-shaped bacillus divided by transversal fission and that reproduction did not proceed via a granule phase. Then what part do these granules play? Can they develop into rod-shaped bacilli?

In many microcultures seeded from seven to fourteen days' cultures in Dubos' medium I have followed these granules from day to day (the number per field varied from a few to twenty or thirty) until the growth of the rod-shaped bacilli became diffuse, and in not one instance did I observe a granule develop into a rod-shaped bacterium, although the germination degree of the rods in the same field was between 90 and 100 %, so that the conditions for growth would seem to be good.

In microcultures I have also followed granules from cultures of various ages, two days to seven weeks, seeded from Dubos' medium, but without seeing any of them germinate; in these experiments, when the growth of the rod-shaped bacteria was slow and failed to become completely diffuse, I was able to follow them for several weeks, in which time some remained unchanged, others decomposed.

In addition, I experimented with two cultures, one four months old, the other seven months, grown in microculture under a cover-glass on serum-tween-mannitol-sauton-agar. Prior to transplanting, the cultures were in visible process of autolysis. On being stained according to Ziehl-Neelsen a number of acid-fast rods were found, most of them granulated, a number of free granules stained by methylene blue, and crowds of granules and amorphous material stained by methylene blue.

On transfer to new medium it was seen that the cultures consisted of masses of granules, some of them very small, and a number of more or less well-preserved rod-shaped cells. During the following days some of the rod-shaped bacteria grew out into colonies in the usual manner, whereas the granules did not change during the whole of the six weeks period of the experiment. Growth among the rod-shaped bacteria at this juncture had long ceased.

How do these granules stain? In young cultures some of them are acid-fast, others are coloured by the counter-stain, methylene blue according to Gram. In older cultures the acid-fastness decreases. As stated, old cultures consist preponderantly of these non-acid-fast Gram-positive granules.

Autolysis.

What is the mechanism of origin of these granules found in cultures of tubercle bacilli? This question is best answered when we observe what happens when the tubercle bacillus autolyses.

Autolysis may be observed in cultures in which the degree of germination is not 100 %. Here it will be seen that cells which do not germinate become less refractile compared with the germinative cells, whose refractive power increases. The difference in this power is often conspicuous even on the day after seeding.

Gradually as these cells become less refractile, more shadowy, one sees granules stand out more distinctly, until at last where the cell has been there is nothing but some granules, from two to six, surrounded by a thin cell membrane, ectoplasm.

In some cases granules can be observed on the outside of the cell membrane owing to its having burst.

If autolysis proceeds further, after two or three weeks in the vicinity of the autolysed cells one may see infinitely small, scarcely visible, faintly refractile, irregular granules, the destruction products of cells and granules. Ectoplasm, and particularly granules, are highly resistant; in several cases I have observed them for one or two months without seeing them change.

I have also observed autolysis in some cultures which I followed for two months. A few of the cells autolysed in the usual manner, becoming less refractile and segmenting into granules.

That granules originate by autolysis is also shown by the fact that old cultures consist chiefly of these elements. On examining an old microculture we find numerous free granules in among the colonies, the products of autolysis with the bursting of the ectoplasm. Most of the granules, however, remain on the site of the rods, making it difficult to see how far advanced autolysis has progressed in a culture of this kind. It is only after seeding on a new medium that we can see how large a share the granules represent.

In microculture I have also studied the »autolysis« or dissolution of a fourteen-day culture treated by »Kochning« for half an hour. Immediately after seeding one sees weakly refractile cells with slightly more refractile granules, very much the same as a living culture. The cells remain practically unchanged, until after three or four weeks they fall into myriads of tiny particles. Here, then, is direct fragmentation of the bacilli without previous granular formation.

A granular transformation of the tubercle bacillus similar to that during autolysis is to be seen under the influence of certain lytic substances, some antibiotica and glycerine. I shall revert to this later.

Filterable Granules.

Just a word about filterable granules. If such granules can grow upon a medium suitable for the growth of tubercle bacilli, as Vaudremer assumed, I would often in my microcultures have observed cells appear at places where I had not previously seen or charted them. This has not been the case, however.

Below I shall describe the effect of streptomycin, penicillin, sulphathiazol and glycerine on the growth of the human tubercle bacillus.

Streptomycin.

In 1944 Schatz & Waksman²⁸ found that streptomycin was bacteriostatic and bactericidal to *Mycobact. tuberculosis*. Concentrations of 200 to 300 units per c. c. medium killed the cells in the course of a few days; lower concentrations had a similar effect in the case of sufficiently long incubation, ten days or more.

Wolinsky & Steenken³⁷, 1947, studied the sensibility of a T.B. strain, H₃₇ Rv, in various media, including Dubos'. The effect on growth was determined partly by visible turbidity, partly by quantitative measuring in a photo-electric colorimeter. The quantity of inoculum was 0.1 c. c. In a concentration of 0.05 µg per c. c. streptomycin caused little growth inhibition, 0.2 µg considerable inhibition, and 0.4 µg complete inhibition. Incubation was for 16 to 20 days, pH 7.1—7.4.

Smith & Waksman³⁰, 1947, studied the effect of streptomycin on a non-pathogenic, human strain and a pathogenic, human strain, H₃₇ Rv. The bacteriostatic effect was tested in fluid Dubos' medium, the bactericidal effect in Dubos' agar. For the non-pathogenic strain bacteriostasis at 0.2—0.3 µg was found to be dependent on the length of the incubation time. With short incubation (6 hours) bactericidal effect required more than 2 µg, with long incubation (240 hours) 0.3 µg. In the case of *Mycobact. tub.* H₃₇ Rv, 2 µg were required for complete inhibition and 1 µg for partial inhibition. Incubation 1 to 14 days.

The main effect on the morphology of the tubercle bacillus was the loss of acid-fastness, increased granulation and, in strong bacteriostatic concentrations, a shortening of the rod.

Own Investigations.

To serum-tween-mannitol-sauton-agar (sauton with glycerine, human serum albumin) was added streptomycin (Merck) in concentrations of 2, 1, ½ and ¼ unit per c.c. (1 unit = 1 µg). From this microcultures were prepared by means of the above-described technique, seeding from an eleven-day culture from Dubos' medium. A control preparation was also made.

Immediately after seeding I picked out the most uniform fields possible in the various slides and plotted them on a diagram. The same cells were then observed by daily microscopy for a period of five weeks. The observations made in the various cultures will be described briefly:

Control culture: Germination 96 %. Apart from a few slightly swollen cells the cells were normal, and diffuse growth was obtained in the course of fourteen days.

Culture with 2 u. streptomycin per c.c. medium: After 24 hours the cells are slightly more refractile, but there was no definite growth. From even the second day it was possible to observe incipient lysis, which increased rapidly. The cells became less refractile, segmented into granules which in two or three weeks broke up into tiny grains, when lysis was complete.

Culture with 1 u. streptomycin: After 24 hours the cells were more refractile, a few had grown in length and thickness and had also curved, but most of the cells were unchanged. Thus there was little primary growth. From the second day the picture remained unchanged. The incipient cell division stopped, there was complete bacteriostasis followed by lysis as with the foregoing concentration.

Culture with $\frac{1}{2}$ u. streptomycin: After 24 hours the refractive power of the cells increased and they had grown in length and thickness; some had curved in the initial fission phase. After the second day there was complete bacteriostasis and lysis had commenced. Lysis proceeded for the most part as already described, but some cells became much swollen during the process, their protoplasm became darker, and granules appeared distinctly. They were much larger than usual, 8 to 9 μ . A few of these cells were still to be seen in the culture at the close of the experiment five weeks later.

Culture with $\frac{1}{4}$ u. streptomycin: The most marked morphological changes were observed at this concentration. In the first 24 hours the refractive power of the cells increased and they grew in length and thickness. In the second and third days this growth continued, while simultaneously fission began accompanied by curving and incipient angle-formation; this, however, was succeeded by bacteriostasis. Only a few cells completed their fission; it was incomplete as far as the great majority was concerned, the rods still forming obtuse angles with one another. There was a prolonged lag-phase, two days, which was not succeeded by a division phase.

In the very next day, the fourth, lysis of some cells was observed and it continued during the following days. It set in first in the cells which had not begun to grow. Cells which had commenced to grow became swollen, some during growth, others during lysis. Some of these swollen cells had a length of 8 to 10 μ , they had a darker protoplasm and distinct granulation. In this phase they were of different shapes: club-shaped, spindle-shaped or like a Pezzer catheter, the swelling taking place a little way from one end. The point of the club corresponded to the site of fission (fig. 5).

Lysis in these cells often set in rapidly, one or two days after they had become swollen; in other cases they remained unchanged for weeks. After two or three weeks lysis was almost complete, the swollen granulated cells decomposed into tiny particles, though some were not altered at the end of the experiment.

The experiment was discontinued at the end of five weeks, there having been only infinitesimal changes during the last two weeks. Transplantations were made from the various microcultures to Dubos' medium, but the only one that gave any growth was the culture with $\frac{1}{2}$ u. streptomycin.

In addition, from the culture with $\frac{1}{4}$ u. streptomycin I transplanted again into microculture on medium without streptomycin. Numerous

granules were then seen and it was also possible to find some of the swollen cells, but they gave no growth within a period of two months.

At the close of the experiment I also made some stained preparations according to Ziehl-Neelsen. A few acid-alcohol fast granulated rods were found in the cultures with $\frac{1}{4}$, $\frac{1}{2}$ and 1 unit streptomycin.

In another experiment of the same kind I examined the effect of $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ and $\frac{1}{64}$ u. streptomycin per c.c. medium. For seeding I again used an eleven-day culture.

Control culture: Germination 94 %. A few swollen cells, otherwise normal growth, diffuse in the course of twelve days.

Culture with $\frac{1}{8}$ u. streptomycin: Germination 86 %. During the first six or seven days there was definitely a slight bacteriostatic effect. The cells were large, some swollen, and several were seen to be in process of lysis. Thereafter growth was rapid and diffuse growth was reached simultaneously with that of the control culture.

Culture with $\frac{1}{16}$ u. streptomycin: Germination 86 %. Several swollen cells and a few cells in lysis, otherwise not different from the control culture.

Culture with $\frac{1}{32}$ u. streptomycin: Germination 97 %. A few swollen cells. Growth almost diffuse already after nine days.

Culture with $\frac{1}{64}$ u. streptomycin: Germination 96 %. A single swollen cell. Growth diffuse after seven days.

The cultures were observed for six weeks after diffuse growth had set in, in order to ascertain whether lysis would occur later, but they differed in no way from the control culture.

With the concentrations of 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ u. streptomycin the experiment was repeated on serum-tween-mannitol-sauton-agar without glycerine and with bovine serum albumin; the result was the same except that the control culture, whose germination percentage this time was 97, contained no swollen cells.

On comparing the results of the experiments it will be seen that in concentrations of 2 and 1 unit per c. c. medium, streptomycin has an almost immediate bactericidal effect followed by lysis.

Concentrations of $\frac{1}{2}$ and $\frac{1}{4}$ u. have a bacteriostatic effect followed by lysis. Lysis is not complete, however.

Concentrations of $\frac{1}{8}$ and $\frac{1}{16}$ u. have a slight, transient bacteriostatic and lytic effect.

Concentrations of $\frac{1}{32}$ and $\frac{1}{64}$ u. have no definite bacteriostatic effect; in fact, the effect is almost growth-promoting.

Penicillin.

Abraham et al.¹ 1941 found no effect from penicillin on the tubercle bacillus in glycerine broth, highest concentration 40 u. per c. c. medium. The penicillin was renewed every other day. The inoculum was large.

Smith & Emmart³¹, 1944, observed no effect from penicillin in glycerine broth and Proskauer & Beck's medium on a virulent human laboratory strain in concentrations up to 30 u. per c. c.

Woodruff & Foster³⁸, 1945, observed no effect from penicillin on a number of mycobacteria, including an avirulent human strain, even with concentrations of 1000 u. per c. c.

Friedmann⁶, 1945, obtained good growth of human, bovine and avian tubercle bacilli in the presence of penicillin in an initial strength of 20 u. per c. c. medium, Tyrode's solution containing tissue from embryo chicks.

Iland⁴³, 1946, found bacteriostatic effect of 20 to 80 u. of penicillin on freshly isolated strains of *Mycobact. tub. hominis*. An avirulent laboratory strain was not inhibited — it destroyed penicillin. Iland employed Pryce's method of cultivating on a slide, modified according to Muller. The medium was an aqueous extract of Löwenstein's medium.

Ungar & Muggleton³⁴, 1946, found that penicillin in low concentrations, 1 to 5 units per c. c. medium, gave an increased growth of a human, virulent strain in liver-extract broth and a modification of Loug's synthetic medium. The effect was due to penicillin and not to impurities.

Own investigations.

Standard penicillin, 1510 units per mg., was added to serum-tween-mannitol-sauton-agar (sauton with $\frac{3}{4}$ % glycerine, bovine serum albumin) to give concentrations of 10, 20, 40 and 80 units per c. c. medium. The inoculum was a twelve-day culture from Dubos' medium. Microcultures were prepared in the usual manner, and simultaneously a control. The most uniform fields possible were selected in the control culture and in the cultures with penicillin, and the cells were observed daily.

Control culture: Germination percentage 95. The cells were normal, and there was diffuse growth in the course of twelve days.

Culture with 10 u. penicillin: From the second day a few large, swollen cells, mostly clubs; similar cells were observed throughout the whole period of growth; some of them lysed. Some cells not morphologically changed were also seen to undergo lysis. After eighteen days growth ceased, but was scarcely diffuse as yet. After two and three months the picture was practically unchanged; compared with the control the cells were not so well preserved.

Culture with 20 u. penicillin: The number of swollen and lysed cells was somewhat higher but still represented only a low percentage. Growth otherwise was as in the culture with 10 u.; there was a slight bacteriostatic and lytic effect.

Culture with 40 u. penicillin: In the first and second days there was primary growth, the refractive power of the cells increased, they grew in length and breadth, and fission began. From the third day there was distinct bacteriostasis. In the fourth and fifth days there were a number of large, club and spindle-shaped, swollen cells; nu-

merous cells were in lysis, others weakly refractile. In the following days there was again more rapid (secondary) growth and small colonies formed, while lysis continued, during which numerous free granules gradually appeared.

After eighteen days growth had ceased; there were small colonies but nothing like diffuse growth; a number of cells were still in lysis, some of them swollen. After two and three months the small colonies were unchanged, but the cells in them seemed to be less well preserved than in the control. Lysis was now complete in those cells that were in lysis and there were myriads of tiny particles from them.

Culture with 80 u. penicillin: In the first and second day it was observed that, as usual, the refractive power of the cells had increased; the cells grew in length and thickness and fission began, the rods becoming curved and forming obtuse angles; on the third day, however, almost complete bacteriostasis set in. The cells stopped in the process of division, some of them swelling and assuming club, spindle or Pezzer-catheter form and becoming much longer, up to 8 or 9 μ .; they had a darker protoplasm and distinct granules.

By the fourth day most of the cells were distinctly lytic, the process being the usual one. The cells became less refractile, granules were more distinct, and finally one observed a weakly refractile ectoplasm, within which lay two to six more refractile granules.

Lysis continued during the succeeding four to eight days, though a few cells divided, including one or two of the swollen ones (fig. 6).

Nine days later lysis was almost complete; a few large and swollen cells remained as well as three or four fairly well-preserved cells as survivals of a small colony. These cells then began quickly to divide and in the following days formed a fair-sized colony of normal cells. Similar cells were seen elsewhere in the slide, and finally macroscopic colonies were formed. It was not ascertained whether this secondary growth was due to penicillin-resistant bacteria or whether the penicillin at this juncture was entirely destroyed.

After eighteen days growth in the colonies ceased and the lysis of the other cells was almost complete.

After two and three months the aforesaid colonies were unchanged, though the cells seemed to be less well preserved than in the control slide. The lysis of the other cells was complete, though their contour could still be perceived. Myriads of tiny particles were now seen in the culture.

The experiment was discontinued after three months. On transplanting to Dubos' medium from the microcultures there was abundant growth after a week in all cultures, but least in the one from 80 units of penicillin.

Stained preparations were also made according to Ziehl-Neelsen and Gram.

When Ziehl-Neelsen stained there were numerous acid-fast rods

in all slides as well as a number of granules coloured by the counter-stain, methylene blue. In the preparations from the cultures with 40 and 80 units of penicillin the acid-fastness was decidedly less than that in the control; the cells were mostly faintly stained, and the methylene blue dominated more in these slides.

When Gram-stained, all the preparations contained Gram-positive rods and granules, but those from the cultures with 40 and 80 u. penicillin were nothing like so well stained.

In another, later experiment with serum-tween-mannitol-sauton-agar (sauton without glycerine, bovine serum albumin) I tested the effect of 1 and 5 units of standard penicillin per c. c. medium. The inoculum was from a 14-day culture.

Control culture: Germination percentage 93. Growth barely diffuse after fourteen days, then stationary. Unchanged after a month.

Culture with 1 u. penicillin: Germination 98 %. Growth otherwise as in the control.

Culture with 5 u. penicillin: Germination 86 %. From the fourth and fifth days there was distinct bacteriostatic effect. There were a number of large spindle and club-shaped, swollen cells with darker protoplasm and distinct granules. Thereafter growth was more rapid and after fourteen days was of the same extent as in the control, but there were still a number of swollen cells, a few in lysis. Unchanged a month later.

When the experiment was discontinued after a month, seeding in Dubos' medium gave growth in every case.

When Ziehl-Neelsen stained the preparation from the culture with 5 u. penicillin contained a number of coarse, swollen cells, but otherwise conditions were the same.

Using the same medium I tested the effect of penicillin in concentrations of 160, 320 and 640 standard units per c. c. medium, the inoculum being from a sixteen-day culture.

Control culture: Germination 98 %. Growth diffuse after twelve days. Cells well preserved after six weeks.

Culture with 160 u. penicillin: After primary growth there was distinct inhibition and incipient lysis from the second day. However, there was still some growth during the next three to seven days, while simultaneously lysis increased, numerous cells were large, 6 to 8 μ ., swollen, spindle and club-shaped with darker protoplasm and prominent granules. After a month there was considerable, but not complete lysis. Secondary growth of small colonies was seen here and there on the preparation.

Cultures with 320 and 640 u. penicillin: In the first day there was primary growth, the refractory power of the cells increased, they grew in length and thickness while curving and commencing to form angles. From the second day there was almost complete bacteriostasis. The cells were again less refractile and in distinct lysis. The latter pro-

ceeded rapidly without cell swelling and was almost complete after four days, although in each preparation there was still a small cell group with a few wellpreserved cells, whereas all isolated cells had lysed. From the sixth or seventh day there was secondary growth of these cells and they grew rather quickly into small colonies. The same thing was observed elsewhere in the preparation. Thus isolated cells were affected more by penicillin than cells in small colonies.

The experiment was discontinued after six weeks. Transplants to Dubos' medium gave growth in every case.

When Ziehl-Neelsen stained it was found as before that the penicillin-affected cells were highly granulated and poorly stained.

It is evident from these experiments that penicillin has a bacteriostatic and bacteriolytic effect upon the tubercle bacillus strain employed. The effect was demonstrated right down to concentrations of 5 units, but pronounced effect is only obtained with concentrations of 80 units and over; primary and secondary growth was still observed when the concentration was 640 units.

The effect of 80 units of penicillin corresponded almost to that of $\frac{1}{4}$ unit of streptomycin.

Sulphathiazol.

Smith et al.³², 1942, observed very effective inhibition with sulphathiazol on tubercle bacilli in concentrations as low as 5 mg % in glycerine broth.

K. A. Jensen & I. Kjær¹⁷, 1942, using a modified Lowenstein medium found total inhibition with 5 mg %, partial with 1.25 mg %. Inoculum: 10⁻⁵.

Muller²⁵, 1944, found good bacteriostatic effect from sulphathiazol in a concentration of 5 mg %; there is some growth even at 40 mg %. Medium: equal parts of water and citrate-blood. Modification of Pryce's method for slide cultivation.

Own investigations.

Serum-tween-mannitol-sauton-agar (sauton without glycerine, bovine serum albumin), to which sulphathiazol was added in concentrations of 1, 2.5, 20 and 40 mg % per c. c. medium. A twelve day culture from Dubos' medium was used for inoculation. Microcultures in the various concentrations as well as a control culture were prepared in the usual manner and observed under the microscope.

Control culture: Germination percentage 92. Cells normal. Growth almost diffuse after thirteen days, then stationary. The cells well preserved after two months.

Culture with 1 mg %: After primary growth, i. e. cells becoming more refractile, growth in length and thickness, incipient division

with curving and angle-formation in the first and second days, there was some bacteriostasis from the third day but still accompanied by some growth. Some cells swelled up and assumed club or spindle shape, during which they increased considerably in size, 8 to 10 μ ., and they had a darker protoplasm and distinct granules. Some of these cells underwent lysis, others after one or two divisions continued growth as normal cells. Growth gradually increased, and after thirteen days there were small and large colonies, but there were still some swollen cells, and a few in lysis. Growth after this was only slight. After two months there was a preponderance of small, weakly refractile cells.

Culture with 2.5 mg %: As with 1 mg %, there were distinct bacteriostasis and lysis from the second and third days. The number of swollen cells was much greater. Isolated cells were affected most, cells in small colonies less so; within a colony the peripheral cells were more affected than those more centrally. Growth otherwise as above.

Culture with 20 mg %: No definite growth. Granular segmentation of numerous cells observed already after one day, and after two days numerous cells were distinctly lytic and the process accelerated rapidly. In most cases lysis took place without change of form, but some cells swelled considerably in the process. After two or three weeks lysis was complete except for a few large cells with darker protoplasm and distinct granules, and myriads of tiny particles were now seen in the preparation. After two months the picture was practically unchanged.

Culture with 40 mg %: No primary growth. Distinct granular segmentation after one day, rapidly accelerating lysis without morphological change. After two or three weeks lysis was complete and there were numerous small irregular particles.

In another experiment I tested the effect of concentrations of 5 and 10 mg %. A fourteen-day culture from Dubos' medium was used for inoculation.

Control culture: Germination percentage 95. Cells normal. After two or three weeks growth stopped, but was not wholly diffuse. After two months the cells were well preserved.

Culture with 5 mg %: After primary growth in the first and second days, the third day saw complete bacteriostasis and incipient lysis. The growth of many cells ceased right in the process of fission. Many cells swelled considerably, up to 8–10 μ . in length, and assumed club, spindle or Pezzer-catheter form; they had a darker protoplasm and distinct granules (fig. 7). Lysis was slow, and slowest in the swollen cells; after two or three weeks there were myriads of tiny particles. After two months there were still a few swollen cells; all seemed to be damaged; those best preserved were in large groups.

Culture with 10 mg %: Conditions in the main as in the culture with 5 mg %.

The experiments were discontinued at the end of two months. Inoculation in Dubos' medium gave growth at the concentrations of 1, 2.5 and 5 mg %, but not at the other concentrations. When stained as per Ziehl-Neelsen the preparations from the cultures with 1, 2.5, 5 and 10 mg % were found to contain acid-fast rods, most of which from the cultures with 5 and 10 mg % were swollen spindle or club-shaped, highly granulated and weakly stained.

These experiments show that in concentrations from 1 to 20 mg % per c. c. sulphathiazol has an increasing bacteriostatic and bacteriolytic effect on the tubercle bacillus E_5 . Concentrations from 20 to 40 mg % are bactericidal.

5 mg % sulphathiazol corresponds roughly to $\frac{1}{4}$ unit of streptomycin.

Glycerine.

As a component of media for the cultivation of tubercle bacilli, glycerine was introduced by Nocard & Roux²⁶ in 1887. Since then it has been employed in several different media, usually in a concentration of from 2 to 5 %; it is often regarded as a necessary component, and it has been used for separating the human and the bovine types (Th. Smidt²⁹). Löwenstein²¹ in 1930 described his medium as containing $\frac{3}{4}$ % glycerine, and later in 1931²² 6 %.

Of these media, K. A. Jensen¹⁵, 1932, found that the former with $\frac{3}{4}$ % glycerine was the better, as the high glycerine addition in the latter medium inhibited the growth of the bovine strain. Subsequent studies by the same worker¹⁶ have shown that $\frac{3}{4}$ % glycerine is the optimal concentration for the human and bovine strains, though certain bovine strains grow best on Löwenstein's medium without glycerine.

Own investigations.

In my first experiments with serum-tween-mannitol-sauton-agar I often observed that in the microcultures there were large, club or spindle-shaped, swollen cells, some of which underwent lysis, others developed into normal cells after one or two divisions. The cause of the occurrence of these cells was long a problem to me.

However, I made a comparison of the growth in fluid serum-tween-mannitol-sauton medium with that in Dubos' medium and found similar cells in the former; moreover, the number of granules in this medium was greater than in Dubos' medium. Serum-tween-mannitol-sauton medium evidently had a bacteriolytic effect on the tubercle bacillus.

On comparing the content in serum-tween-mannitol-sauton medium with that in Dubos' medium I found that this effect was probably due to glycerine, which was the most conspicuous difference between the two media.

That glycerine actually is bacteriolytic for the tubercle bacillus will be seen from the following experiment:

Serum-tween-mannitol-sauton-agar was prepared without glycerine and also with glycerine in various concentrations. Microcultures were prepared and observed with the microscope.

First I studied the effect of 2 and 6 % glycerine (the same concentrations as in Sauton's medium). The inoculum was a twenty-one day culture from Dubos' medium.

Control culture: Germination percentage 98 %. All cells slender, thin, granules not specially prominent. Diffuse growth after six days.

Culture with 2 % glycerine: The cells as a whole rather coarser, some large, swollen, granules more prominent, more refractile, presumably on account of greater pigment formation on the glycerine medium. Growth diffuse after eight days.

Culture with 6 % glycerine: In the second to the fourth day definitely little bacteriostasis, the cells large on the whole, a few club-shaped, swollen, with darker protoplasm and distinct granules; one of the latter lysed, while others developed after one or two divisions into normal cells. Granules prominent. Growth diffuse after nine days.

In another experiment the effect of $\frac{3}{4}$ (the same concentration as in Lowenstein's medium), 10, 20 and 30 % glycerine was tested. An eight-day culture from Dubos' culture was used for inoculation.

Control culture: Germination percentage 96. The cells thin with non-prominent granules. Growth diffuse after seven days. After ten weeks the cells were fairly well preserved.

Culture with $\frac{3}{4}$ % glycerine: Cells again thin. From the third day there were a very few club-shaped, swollen cells, one of which lysed. Growth diffuse after seven days, hardly so homogeneous as in the control culture. The cells were more refractile and granules more prominent as a consequence of pigment-formation. A number of free granules gradually appeared. The cells fairly well preserved after ten weeks.

Culture with 10 % glycerine: After primary growth in the first and second days, during which they became more refractile, they grew in length and thickness and fission began with curving and obtuse-angle formation; there were distinct bacteriostasis and incipient lysis from the third day. The cells were very large, 8—10 μ ., uniformly swollen or in club, spindle or Pezzer-catheter form, with darker protoplasm and distinct granules.

In the following days there was a very slow growth with constant swelling, while lysis increased; gradually the lysed cells were seen as shadows with slightly more refractile cytoplasm and granules (fig. 8).

After three weeks, apart from numerous cells in lysis and free granules, there were still a number of fairly well preserved, slightly swollen cells. Some of these, which had lain in a small colony, now began rapid division and secondary growth, and in the course of the

next fortnight or so they grew out into rather large colonies consisting of fairly large, 4—8 μ ., evidently glycerine-resistant cells. Similar cells were seen everywhere in the preparation, while at the same time the lysis of the other cells continued and became almost complete.

After ten weeks there were colonies of resistant cells, numerous cell shadows, and in between them tiny particles as well as a few swollen cells.

Culture with 20 % glycerine: No primary growth. Bacteriostasis and incipient lysis observed after one day. Simultaneously with increasing lysis there were after a few days a few slightly swollen cells with darker protoplasm and distinct granules. These cells lysed at a slower rate than the others. After three or four weeks there were numerous tiny particles as signs of disintegrating granules.

After ten weeks lysis was almost complete, though there were still a few swollen cells.

Culture with 30 % glycerine: At this concentration there was complete bacteriostasis already in the first day. Lysis proceeded without the cells swelling. After two weeks there were numerous small particles, and lysis was complete after three or four weeks.

Ziehl-Neelsen staining at the end of the experiment: Control culture and $\frac{3}{4}$ % glycerine: Numerous acid-fast rods. 10 % glycerine: a few acid-fast rods, some large, swollen and highly granulated, numerous rods and granules stained by methylene blue. 20 % glycerine: a very few acid-fast rods, otherwise granules and amorphous material stained by methylene blue. 30 % glycerine: No acid-fast rods. Granules and amorphous material stained by methylene blue.

The experiments showed that glycerine in high concentrations, 20 to 30 %, has a bactericidal effect on the tubercle bacillus *E.*. Lower concentrations are bacteriostatic and bacteriolytic, distinctly so in concentrations 6 to 10 %, but the effect is demonstrable right down to $\frac{3}{4}$ %.

10 % glycerine induces morphological changes almost corresponding to the effect of $\frac{1}{4}$ unit streptomycin.

On comparing these observations with the diphtheroid rods, ovoid corpuscles and fungoid cells found by Reenstjerna²⁷⁾ and Hollström¹²⁾ in glycerine broth, one wonders whether these forms are not merely morphological variants of the tubercle bacillus produced under the influence of glycerine.

The glycerine-affected, large, spindle-shaped, swollen tubercle bacilli which I have described above are very like yeast or oidium cells and quite like bacilli which I have found in deep cultures of *E.* in 4 % glycerine broth (and also in fluid serum-tween-mannitol-saulon, which contains 6 % glycerine), just as I have been able in the first days after transplantation to demonstrate by Ziehl-Neelsen staining non-acid-fast, highly granulated (diphtheroid) rods stained by methylene blue.

Discussion.

A comparison of the effects of the various substances tested (streptomycin, penicillin, sulphathiazol and glycerine) on this particular strain of tubercle bacillus shows that these effects are fundamentally the same.

Strong concentrations of the substances cause bactericidi followed by lysis, without special morphological changes in the cells, except for the morphological changes that always accompany lysis.

In lower concentrations the substances induce bacteriostasis and bacteriolysis, accompanied by considerable morphological changes, swelling and changes of form in the cells. The greatest morphological variations result from those concentrations which just permit of growth or allow slow growth under unfavourable conditions.

What is the nature of the effect of the low concentrations on the growing cell? The higher concentrations cause the direct killing of the bacilli.

For penicillin it is stated, *inter alia* by Fisher⁴, 1946, that an influence is brought to bear upon the fission mechanism of the cell, an influence on one or other phase in the cell metabolism; as evidence of this Fisher points out that penicillin is most effective on the rapidly growing organism, whereas it does not affect bacteria in the resting phase.

That the fission mechanism is affected is clear from my experiments, in which the cell growth was often seen to stop midway in the process of division.

Then how is the fission mechanism or metabolism affected? In experiments with microcultures in which access of air to the cultures was prevented by a special device, I have seen exactly similar morphological changes to those produced by antibiotics and glycerine, and therefore I considered it likely that these substances affect cell respiration.

The following is a brief description of an experiment of this kind: Serum-tween-mannitol-sauton-agar was inoculated with a fourteen-day culture from Dubos' medium, and a microculture was prepared. Air was prevented from entering by placing a cover-glass on the underside of the agar block and sealing it airtight with molten paraffin to the cover-glass on the upper side of the block, the cover-glass which forms the ceiling of the chamber. A control culture was also prepared.

Control culture: Germination percentage 92. Cells normal. Almost diffuse growth in the course of eighteen days, then stationary.

Culture sealed from air: During the first and second days the cells became more refractile, they grew in length and thickness, and fission commenced with the rods curving. In the third and fourth days there was bacteriostasis and incipient lysis; numerous cells had ceased to grow midway in the fission process, they swelled up, there were club, spindle and Pezzer-catheter forms, the swollen cells were large, 6—8 μ .

had darker protoplasm and distinct granules (fig. 9). Lysis increased rapidly during the succeeding days.

After two or three weeks there were numerous lysed cells and also a number of swollen cells.

After three months the picture was practically unchanged, though the surviving cells were weakly refractile. Lysis was not complete, oxygen being necessary for this process. Growth was obtained on transplanting to Dubos' medium.

The morphological changes induced by a reduced supply of air (O_2) (the supply of air is not wholly interrupted, as the agar contains air) were the same as those observed from the influence of small concentrations of streptomycin, penicillin, sulphathiazol and glycerine. The natural assumption therefore is that the effect of these substances is due to influence on cell respiration.

Summary.

The normal growth of a human tubercle bacillus strain was observed in microculture, directly under a cover-glass in a moist chamber on serum-tween-mannitol-sauton-agar. It is pointed out that reproduction proceeds by means of transversal fission. Branching occurs very rarely. Granules arise from the autolysis of the rods; they do not develop again into tubercle bacilli.

On the strain employed, streptomycin in concentrations of 1 to 2 units (1 unit = 1 μ g) per c. c. medium has a bactericidal effect. Concentrations of $\frac{1}{4}$ to $\frac{1}{2}$ unit are bacteriostatic and bacteriolytic. Lysis proceeds accompanied by swelling and changes of form in the cells. Lower concentrations, $\frac{1}{10}$ to $\frac{1}{8}$ unit, have little bacteriostatic and bacteriolytic effect.

Penicillin is bacteriostatic and bacteriolytic. The effect is not considerable below 80 units, but it can be demonstrated down to 5 units. Lysis proceeds accompanied by swelling and formchanging. Primary and secondary growth still occur at a concentration of 640 units.

Sulphathiazol is bactericidal in concentrations of 20 to 40 mg %. Concentrations of 1 to 10 mg % are bacteriostatic and bacteriolytic. At these concentrations lysis proceeds accompanied by swelling and form-changing.

Glycerine is bactericidal in concentrations of 20 to 30 %. At $\frac{3}{4}$ to 10 % it is bacteriostatic and bacteriolytic; here again lysis proceeds accompanied by swelling and form-changing.

As similar morphological changes can be produced by a reduced supply of air (O_2), the author considers it probable that the effect of these substances is due to an action on the respiration of the cells.

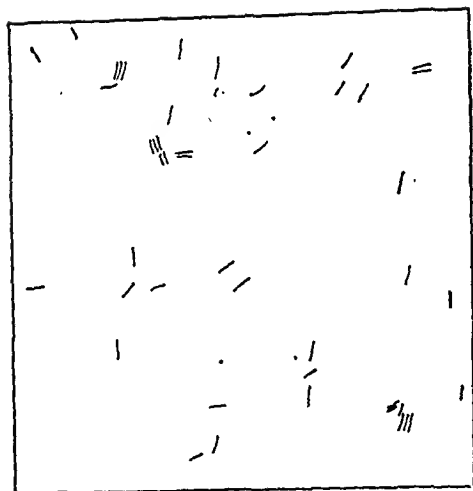


Fig. 2.

Normal cells immediately after inoculation. The cells are faintly refractile with slightly rounded ends and not very prominent granules.

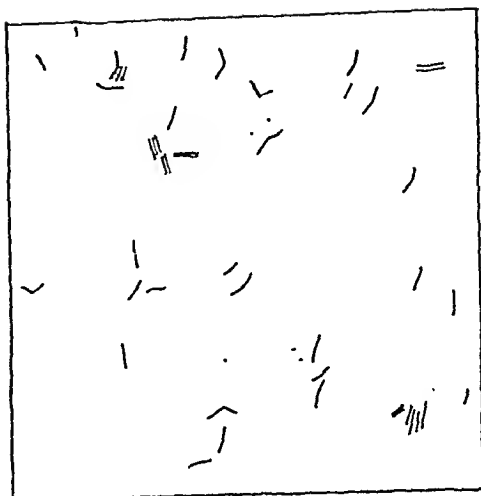


Fig. 3.

Normal cells after one day (same field as fig. 2). The cells more refractile and have grown in length and thickness, some having curved. Granules more prominent. Fission commencing, accompanied by angle-formation.

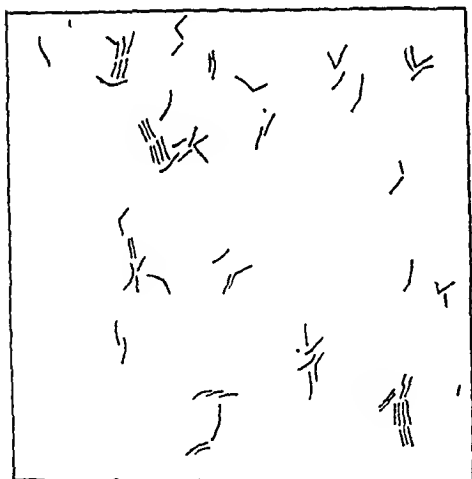


Fig. 4.

Normal cells after two days (same field as fig. 2). Lively fission, partly with angle-formation. Incipient colony formation. The free granules unchanged.



Fig. 5.

Culture with $\frac{1}{4}$ unit streptomycin after five days. Numerous large swollen cells with darker protoplasm and distinct granules. Some cells (punctate; actually they are seen as a row of granules surrounded by a faintly refractile ectoplasm) in lysis.

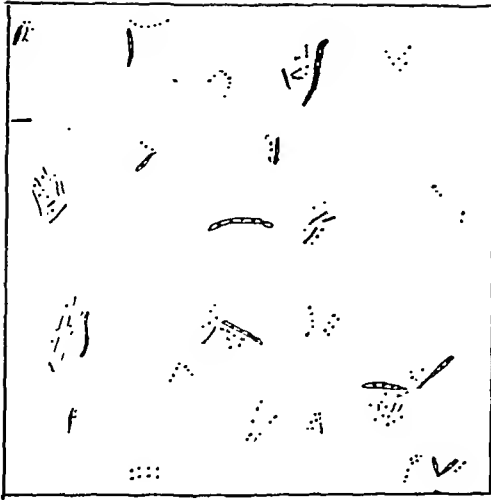


Fig. 6.

Culture with 80 units penicillin after five days. A number of large, swollen cells with darker protoplasm and prominent granules. Numerous cells in lysis.

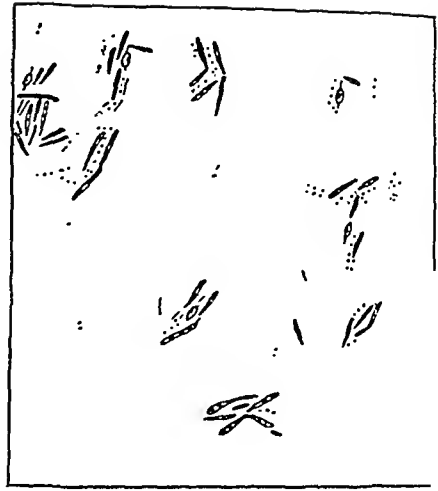


Fig. 7.

Culture with 5 mg % sulphathiazol after five days. Numerous large, swollen (club, spindle and Pezzer-catheter shaped) cells. Some cells in lysis.



Fig. 8.

Culture with 10 % glycerine after 13 days. Numerous swollen cells of various shapes with darker protoplasm and distinct granules. Many cells in lysis.



Fig. 9.

Culture sealed from air, after eight days. Numerous swollen cells and some cells in lysis.

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ELECTRON MICROSCOPE STUDIES OF BREAST- CANCER EXTRACTS OF MICE.*)

By Johs. Dossing, Hans Fr. Helweg-Larsen and Hans Rahbek Sorensen.

(Received for publication October 12th, 1948.)

Bittner's studies on a cancer-inducing factor in milk and different tissues of mice belonging to high-breast-cancer strains, together with *Barnum, Ball, Bittner & Visscher's* experiments on the biochemical and physical properties of the factor has inspired *Passey, Dmochowski, Astbury & Reed* to use electron-micrography in order to show the factor as particles.

Using extracts of lactating breast tissues and of breast tumour tissues of high-cancer strains, the last-mentioned authors found approximately spherical particles of about 200 Å in diameter. Extracts of lactating breast tissues and tumours induced by methylcholanthrene from mice of lower-cancer strains were found to be free from such particles. *Graff, Moore, Stanley, Randall & Haagensen* found similar conditions in milk of a high- and a low-cancer strain of mice, and these results were later verified by *Passey et al.*

In order to confirm the observations of *Passey et al.* in our high-breast-cancer strain (dlb, dilute brown) and in our low-cancer strain (B, a strain with recessive pituitary dwarfism) extracts of tumour tissues and normal lactating breast tissues were prepared after *Passey's* method with minor modifications: Dried tissues were treated with petroleum ether, extracted with distilled water, treated with trypsin for 30 min. at 38° C., and centrifuged for 15 min. at a speed equivalent to 3000 times gravity. The supernatant fluid was examined in the electron microscope.

*) The preparation of extracts was performed in the Biological Institute of the Carlsberg Foundation, Copenhagen (Chief: A. Fischer, M. D.). Messrs. R. S. Page, C. I., and Sv. Dossing, C. I., have kindly rendered us technical assistance and lent us the electron microscope (Metrovick EM 3) during the British Exhibition in Copenhagen 18/9 — 3/10 1948.

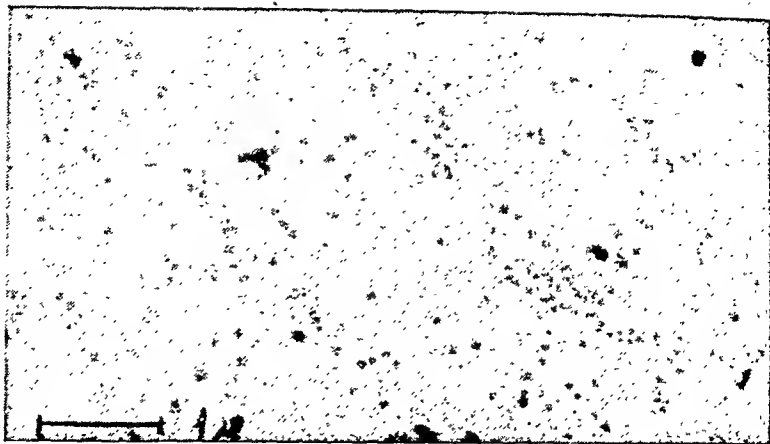


Fig. 1.

Results: All breast tumour extracts from high-cancer mice were found to contain spherical particles, shown in Fig. 1. The magnification is about 15,000 times, and the size of the particles varies from 200 Å to 900 Å. Extracts of lactating mammary tissues from normal low-cancer mice were either found to be free from such particles, or corpuscles identified as contaminations were seen. Only one of 20 control specimens showed corpuscles similar to the particles in tumour extracts, but somewhat larger (Fig. 2).

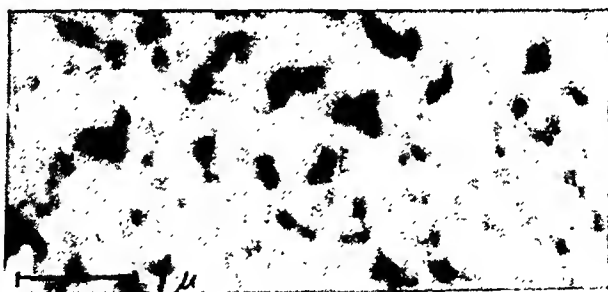


Fig. 2.

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FLUORESCENCE STUDIES ON CARCINOGENS DISSOLVED IN POLYETHYLENE GLYCOLS

By Kai Setälä.

(Received for publication September 25th, 1948.)

The localization and distribution of externally applied carcinogenic hydrocarbons in the living organism have been studied during recent years. These investigations have been especially based upon the fluorescence of the carcinogenic hydrocarbons; the number of publications concerning this subject is, however, comparatively small. It became evident, already in the early work on carcinogenesis, that the nature of solvents played an important role in the results obtained. Since the carcinogenic hydrocarbons are nearly insoluble in water but soluble in organic solvents and oils, in the fluorescence studies — on skin carcinogenesis — certain fat solvents (benzene, chloroform, acetone, etc.) have been employed.

However, it would be valuable, in some cases, if carcinogenic hydrocarbons could be applied in various solvents, both volatile and non-volatile, which had different chemical qualities. In that way it should be possible to get further information as to how the carcinogenic hydrocarbons act in skin carcinogenesis.

Since 1945 the writer has made experiments concerning the histological localization and distribution of carcinogenic hydrocarbons in living cells. The question of whether it would be possible, directly or indirectly, to show if these compounds act at a certain phase of the nuclear cycle was especially interesting.

In another connection the writer has presented some preliminary observations on tumour production brought about by using certain *polyethylene glycols* as solvents and spreaders for aromatic carcinogenic hydrocarbons (13). Here we only refer to the paper mentioned above. The present paper gives an account of the fluorescence studies carried out, using »Carbowax» 1500 (trade mark for certain polyethylene glycols) and *dioxane* (diether of glycol) as solvents and carriers for the carcinogenic hydrocarbons.

Review of literature.

Concerning the solvents used in the present work, the writer, as far as he knows, is the only one to have used these compounds as carriers for carcinogenic aromatic hydrocarbons (11, 12, 13).

Mitchell and Hamner (10), for instance, used polyethylene glycols as carriers for certain growth-regulating substances on kidney bean plants. These compounds readily dissolve the substances mentioned and serve as efficient spreaders when in aqueous solutions.

Wilson, DeEds and Cox (18) when studying the carcinogenic activity of 2-acetaminofluorene, dissolved the carcinogen in propylene glycol. They mentioned that this glycol may in itself have carcinogenic potency also: leukemia developed in a number of rats given propylene glycol subcutaneously. This substance and related compounds have been studied somewhat extensively and have not been considered to be very toxic (7, 8, 16).

As far as the writer is aware no previous work has been reported on the localization, in skin carcinogenesis, of fluorescent carcinogenic hydrocarbons dissolved in polyethylene glycols. *Miller and Bauman* (9) when studying the factors that alter the fluorescence of hydrocarbons (in solutions) mentioned that the fluorescence was most intense in dioxane and in some other solvents. They, as well as *Weil-Malherbe* (17) who also used glycol, say that the chemical composition of a solvent has no relation to the intensity of fluorescence of hydrocarbons dissolved in it, but this was dependent upon the aerated solvent in which the hydrocarbon was dissolved. To this as well as to certain problems related to it we will return in another connection (3).

Concerning the actual histological localization of the carcinogenic hydrocarbon in mouse skin, *Graffi* (4, 5, 6) was apparently the first to use the fluorescence microscope technique to study the accumulation of carcinogens in living cells*).

Summarizing the investigations in which benzene or acetone have been used as solvent and carrier of carcinogen:

(1) Fluorescence of the normal untreated mouse skin: this fluorescence is very weak in general, but sufficiently evident that one can readily identify the major structures within it. The upper portion of the epithelium has a very intense fluorescence of a deep blue colour. The same blue fluorescence is seen in the epithelium of the hair follicles and the sebaceous glands. The fluorescence of the subcutaneous fatty tissue is very weak. Some fat appears to have no fluorescence at all and other droplets vary from blue to green, etc. (6, 14, 15).

(2) Immediately after the first painting: a blue-violet fluorescence, usually less brilliant than that of the sebaceous glands, is encountered in some of the fat cells.

*) The writer did not have the strongly fluorescent benzpyrene. Because of this, both in this review and in the present report, the exact localization of the carcinogen in the tissue is not discussed.

(3) Animals killed up to 24—48 hours after painting: the general appearance, in general, the same as in the foregoing paragraph. — The only change appears in the distribution of blue-violet fluorescence in the subcutaneous fat (14, 15). After about 24 hours some fluorescence is usually apparent throughout the fatty layer, though the superficially situated cells are at that time still the most brilliantly fluorescent.

(4) Animals killed after two to six days: the fluorescent fat cells tend to become evenly distributed throughout the cell-layers and at the same time become gradually less brilliant.

(5) Animals killed after multiple paintings: the carcinogen passes directly into the cytoplasm, but not into the nuclei (1, 2, 4, 5, 6, 14, 15). — Epithelial cell permeability is apparently significantly modified by such an exposure, so that the carcinogen in its lipid solvent can now enter freely. A previously untreated epidermis has never been seen to take up fluorescent material directly. After multiple paintings a slow diffusion takes place into deeper fatty layers, from which it fades slowly (14, 15).

Material and Methods.

The group of animals used in this work included approximately 100 mice of mixed strain. Carcinogen was applied as a paint or in drops, and the animals were killed at intervals of from a few minutes to five days after the first and repeated treatment respectively.

As solvents the following substances were used:

- (1) reagent grade acetone (Merck, Darmstadt).
- (2) »Carbowax« 1500 (Carbide and Carbon Chemicals Corporation, N. Y.), and
- (3) dioxane (L. Light & Co., Ltd., Wraysbury, Middx.).

As aromatic carcinogenic hydrocarbons the following compounds (Eastman Kodak Company, Rochester, N. Y.) were used:

- (1) 20-methylcholanthrene,
- (2) 1.2.5.6-dibenzanthracene,
- (3) 9.10-dimethyl—1.2-benzanthracene, and
- (4) 1.2-benzanthracene.

The application of carcinogen was as drops in the cases of dioxane and acetone (0.3—0.5 per cent of carcinogen), and by painting with unmelted »Carbowax« 1500 (0.25—1.0 per cent of carcinogen).

The tissues were fixed in a 10 per cent solution of neutral formalin, and cut on the freezing microtome at 10 to 20 microns thickness. Another series of preparations was stained with ordinary stains and/or Sudan III.

The preparations were studied with a fluorescence microscope, type Reichert Lux U V.

Results.

Fluorescence of the normal mouse skin. — Concerning the fluorescence of the normal, untreated skin, nothing new was noticed in the present work compared with the review of the literature.



Fig. 1.

Fluorescence of mouse skin 40 minutes after painting with 0.25 per cent methyleholanthrene dissolved in »Carbowax«. The horny layer as well as the sebaceous elements have a very strong pale-blue or dark-blue fluorescence. The lowest layers of the epidermis, on the contrary, have blue-violet fluorescence. No fluorescent material observable in the nuclei.

Fluorescence after a single application of carcinogen. — (a) Dioxane as solvent: on using this substance as solvent and carrier for hydrocarbons, the microscopical picture resembled that when acetone is used as solvent instead of dioxane. The fluorescent material accumulated (immediately after the application) in the horny layer of the epidermis and in the sebaceous glands and their contents. The colour of the fluorescence in the organs mentioned was pale-blue and the intensity of the fluorescence was very strong. The nuclei appeared as optically empty spots in the sebaceous elements. The subcutaneous fatty tissue was not, in general, stained by the fluorescent material used when investigated a short time after application. Only after 1 to 2 hours one could observe fluorescent granules in the subcutaneous fatty layer. When stained with Sudan III it appeared that the fluorescent substance (*i. e.*, changed or unchanged carcinogen) had been distributed in about the same fashion — in general in the lipid material — as the fat stain used. The microscopical appearance was

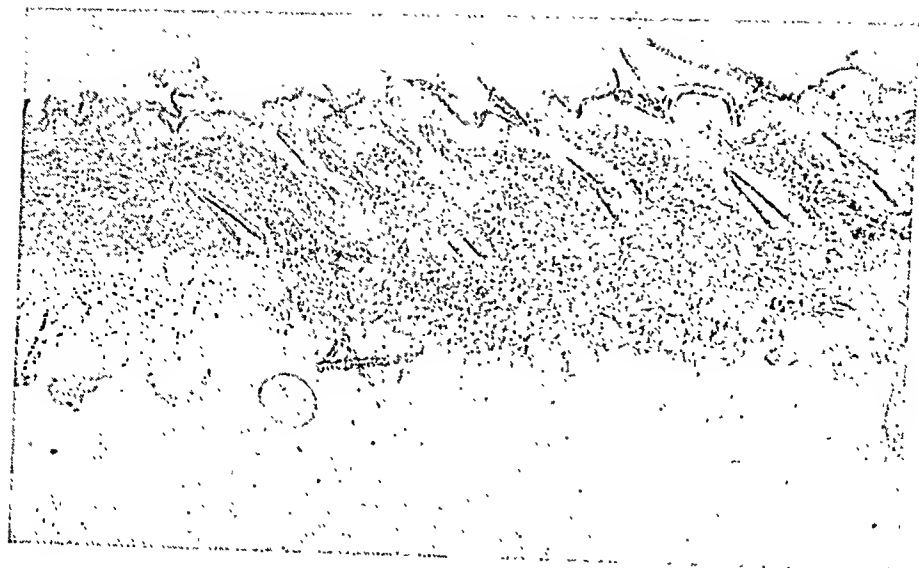


Fig. 2.

Unstained section of mouse skin. The animal killed immediately after the third application of 1.0 per cent methylcholanthrene in »Carbowax«.

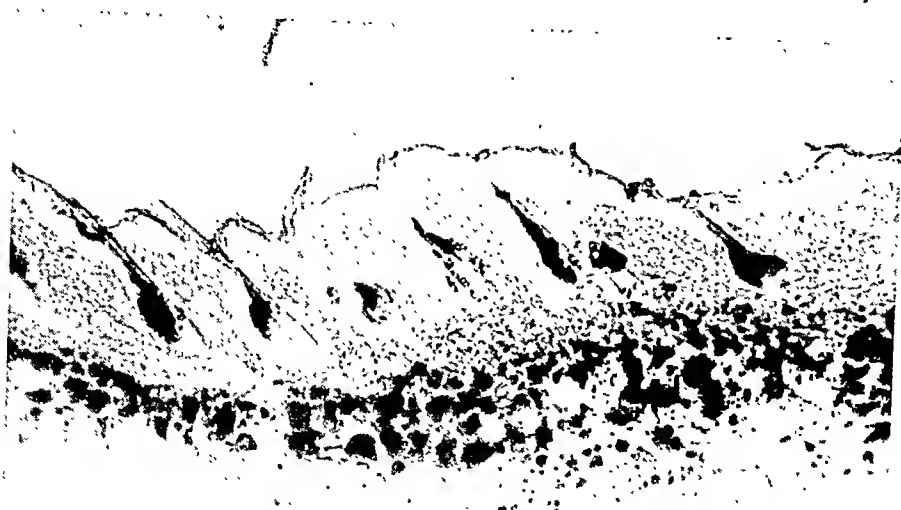


Fig. 3.

The same section stained with Sudan III.

about the same as when acetone had been used as solvent for the carcinogen.

(b) »Carbowax« 1500 as solvent (without melting it before painting): when this organic compound (at the same time non-volatile and readily soluble in water) was used as solvent and carrier for the carcinogenic hydrocarbons, the microscopical appearance was a little different. Using *e. g.*, 0.25 per cent methylcholanthrene as carcinogen the histological figure was as follows (Fig. 1 gives an illustrative example of this): in the first place, there was about 40 minutes after



Fig. 4.

The same preparation, unstained section, photographed in ultraviolet light.

the application no crystalline hydrocarbon at the surface of the skin (but all the carcinogen seemed to have been dissolved in the solvent). The intensity of the fluorescence was very strong in the superficial horny layer of the epidermis and in the sebaceous elements as well as in the most superficial layers of the subcutaneous fatty tissue. The colour of this material was pale-blue to dark-blue, but not blue-violet or violet. An early change — thus differing from the state of affairs when using acetone or dioxane as solvent — in the microscopical localization sometimes appeared in the distribution of the fluorescent material in all layers of the epidermis already some hours after the application of the carcinogen. Even the lowest layers of the epidermis showed blue-violet fluorescence, though comparatively weak. The nuclei were, however, optically empty. It seemed that the carcinogen, dissolved in »Carbowax« 1500, could now enter the epithelial cells, the intercellular spaces and other elements comparatively freely.

Fluorescence after repeated application of carcinogen. — (a) Dioxane as solvent: the microscopical appearance was about the same as in the case of acetone as solvent for the hydrocarbon.

Fig. 3.
High magnification. The microphotograph shows the distribution of carcinogen in «Carbowax».



(b) »Carbowax« 1500 as solvent: mice killed immediately or a few minutes after the last application of carcinogen dissolved in this compound showed yellow-green fluorescence at the surface of the epidermis (*i. e.*, crystalline carcinogen). One conspicuous fact was that the sebaceous glands were, in general, well comparatively preserved after about three to six days from the beginning of treatment.

Figs. 2, 3 and 4 give an illustrative picture of the conditions in the epidermis and in the other tissues after three successive applications of 1.0 per cent methylcholanthrene dissolved in »Carbowax« 1500. The



Fig. 6.

Mouse skin photographed in ultraviolet light about 7½ hours after the death of the animal. 0.5 per cent methylcholanthrene dissolved in acetone.

skin of the mice was painted without melting the carrier before it was applied. Fig. 2 represents an unstained frozen section of the skin, and Fig. 3 was photographed after the same section had been stained with Sudan III. This figure shows the distribution of Sudan positive material in the skin. Comparing Fig. 4 with the foregoing, the following details are notable: at the surface of the epidermis there are only a few hydrocarbon crystals compared with those seen after carcinogen-acetone treatment. The horny layer of the epidermis contains a great amount of material which has a pale-blue or dark-blue fluorescence. The fluorescence of the sebaceous elements — which are well preserved — has about the same colour and intensity. The superficially situated fatty droplets in the subcutis have accumulated fluorescent material of pale-blue and/or dark-blue fluorescent colour. On the other hand, the deeper layers of the epidermis as well as the deeper layers of the subcutaneous fat have a fluorescence of blue-violet or violet shade. As in the case of a single application (see Fig. 1) the

nuclei appeared as optically empty spaces after the third painting also. Fig. 5 represents a higher magnification of mouse skin treated with methyleholanthrene dissolved in »Carbowax« 1500.

In Figs. 6 and 7, taken after the death of the animals painted during life, it is obvious that the localization, intensity and colour of the fluorescent material are about the same both using »Carbowax« 1500 (Fig. 7: 1.0 per cent benzantracene as carcinogen) and volatile acetone (Fig. 6: 0.5 per cent methylcholanthrene as carcinogen). The microphotographs are taken from an unpublished paper by the writer.



Fig. 7.

1.0 per cent benzantracene dissolved in »Carbowax«. Other factors the same as in Fig. 6. (Note the same distribution of the fluorescent material in these figures.)

The exact intraeytoplasmic localization in the tissues of the carcinogen dissolved in polyethylene glycols is not described here. In the literature there are differing opinions concerning the phenomenon mentioned. To this problem we will return in another connection.

Summary.

Certain polyethylene glycols (»Carbowax« 1500, and dioxane) have been used as solvents and carriers for carcinogenic hydrocarbons (20-methyleholanthrene, 1.2.5.6-dibenzanthracene, 9.10-dimethyl-1.2-benzanthracene, and 1.2-benzanthracene).

On using dioxane the microscopical appearance seemed to be about the same as in the case of acetone.

When the non-volatile »Carbowax« 1500, which is readily soluble in water, was used as carrier the carcinogen presumably to permeate the tissues as easily as the carcinogen dissolved in volatile acetone.

After three successive paintings with carcinogen dissolved in »Carbowax« 1500 the fluorescent material was observable in all subcutaneous fatty layers also. The superficially situated fatty droplets had a pale-blue and/or dark-blue fluorescence, and the droplets of the lower layers a blue-violet or violet colour.

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SEROLOGICAL STUDIES ON GONOCOCCI. II. CROSS-ABSORPTION EXPERIMENTS AND FACTOR SERUM DETERMINATIONS.

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In Sections 1 and 3 of the preceding paper, a description is given of the technique employed in complement-fixation tests and absorption experiments. It is pointed out that absorption is required for differentiation of the gonococci into »types«. As the preliminary cross-absorption experiments had not yielded two serologically identical strains, it seemed obvious to investigate first the possibility as to whether or not serologically identical strains really exist. To begin with, rabbit sera were used for these experiments and later guinea-pig sera.

1. Preliminary Experiments with Strains which, according to their Origin, might be expected to be Serologically Identical (Rabbit Sera).

Such strains might chiefly be found by making repeated cultures from the same patient or from a series of contacts. Further, a condition for the reliability of the type diagnosis of a given gonococcus strain (and for epidemiological serviceability of the diagnosis) would naturally be that strains isolated successively from the same patient would prove serologically identical. Also strains from two or more patients in a »chain infection« (series of contacts) should resemble each other, but even this contingency implies some factors of uncertainty.

Previous investigators have not occupied themselves very much with the question of the identity of strains which, according to their origin, presumably should be identical. Vollmond (1923) mentioned that strains from married or courting couples, or sibs, were always of the same type, but he fails to state how many strains in this category

he has actually examined. He further states that he has never found two quite identical strains. Atkin (1925) examined 6 pairs of strains isolated from the urethra and cervix of the same patient, and found 5 pairs to be identical, whereas one pair showed two different types — from the urethra and from the cervix. Finally, Uroma (1943) states that two strains from a married couple were serologically identical.

Therefore, a number of strains in the above-mentioned category were collected, viz. 6 »chain infections« (15 strains from 14 patients) and 50 »sets of strains« (120 strains from 50 patients). The chain infections were designated as $A_1 - A_2$, $B_1 - B_2$, while the sets of strains were labelled 1a—1b, 2a—2b—2c, 3a—3b

The antigens corresponding to a certain chain infection comprising 4 strains appeared to be particularly toxic to rabbits, and the titres obtained in the surviving animals were strikingly high.

Torrey (1908) found gonococcal strains to differ greatly in toxicity.

Vollmond (1923) claimed that he had been able to demonstrate that strains giving the highest titres were the most toxic. The present experiments thus appeared to confirm the experiences of Vollmond.

Fourteen out of seventeen sets of strains (i. e. 82 %) proved, on cross-absorption, to be made up of identical — or almost identical — strains. In 4 of the »chain infections«, the strains were found to be identical, while two of the chains consisted of serologically different strains. An example of this is seen in Table 3. Here strain 36a is seen to be serologically identical with strain 36d, but different from strain 36b, which is identical with 36c. The letters a — h — c — d give the sequence of the isolation of the strains, 36a being the first and 36d the last strain isolated.

Altogether 18 series of strains (6 chain infections and 12 sets of strains) were examined by cross-absorption tests without preliminary treatment of the absorbing antigen with guinea-pig serum. In 11 of these series (i. e. 61 %) one or often more sera were rendered anti-complementary by absorption.

Thus, with the technique here employed, it was not practicable to show that strains isolated from the same patient (within the same period of illness) are always identical.

2. Experiments with Factor Sera (Rabbit Sera).

For several years, the so-called »factor sera« have been employed for determination of the antigenic structure in *Salmonella* diagnosis (Kauffmann 1938).

*) Most of these strains were collected by means of the card index of infections kept at the Rigshospital, and thanks are due to Professor E. Haxthausen for permission to make use of this index.

Table 3.

Set of 4 *Gonococcus* Strains examined in Cross-absorption Experiments.
 Absorbing antigen treated preliminarily with: 1) inactivated guinea-pig serum.
 2) buffer saline.

*) 2 rabbits per strain, pooled sera.

		Antigen				Serum-control
		36 a	36 b	36 c	36 d	
Non-abs. serum*)	36a	12	9	9	12	0
Serum abs. with	36a	0	0	0	0	0
	36b	1	0	0	2	0
	36c	0?	0	0	1	0
	36d	0	0	0	0	0
Non-abs. serum	36b	12	11½	10	13	0
Serum abs. with	36a	0	0	0	0	0
	36b	0	0	0	0	0
	36c	0	0	0	0	0
	36d	0	0	0	0	0
Non-abs. serum	36c	11½	10	11	10	0
Serum abs. with	36a	0	0	0	0	0
	36b	0	0	0	0	0
	36c	0	0	0	0	0
	36d	0	0	0	0	0?
Non-abs. serum	36d	15	10	10	15	0
Serum abs. with	36a	0	0	0	0	0
	36b	6	0	0	6	0
	36c	6	0	0	6	0
	36d	0	0	0	0	0

Conclusion of absorption experiments: Urethral strains 36a = 36d
 different from: Cervical » 36b = 36c

By the term »factor serum«, Kauffmann means an absorbed serum, the antihody content of which is established through a number of cross-agglutination experiments. Thus a *specific diagnostic serum* is obtained instead of the »non-specific unabsorbed serum«.

Examination of a strain (antigen) with a series of factor sera is called »factor determination« or determination of the »factor formula«. In *Salmonella* and *pneumococcus* diagnostics (Morch 1913) the term »factor« is applied to antigen components as well as to the antibody components. In the present work, the term »factor« will be used in a slightly different way: here the *antigen* components will be designated by means of small letters, and *antibody* components by

means of capitals. The term »factor« will be applied only to the antibody contents of sera.

The aforementioned cross-absorption experiments showed that it was difficult to determine the antigenic structure of gonococci, because, among other reasons, many of the absorbed sera became anti-complementary.

Therefore, an attempt was made to produce some sort of »factor sera«, — i. e. absorbed sera — which were known from experience not to be anticomplementary, and which possessed a certain potency, preferably not under 6 degrees of potency (2 test tubes positive) as measured with the corresponding homologous strain.

The antibody content of some of these factor sera was known to some degree from cross-absorption experiments; others were chosen for the above-mentioned reasons.

Some preliminary experiments showed that strains which, according to their origin, ought to be identical showed, as a rule, the same »factor formula«, while other strains — selected at random — were all different. Several of the latter strains, however, were found to have factor formulas of a »type« similar to several of the sets of strains. Cross-absorption experiments performed with strains having »uniform« factor formula gave only 2 identities, viz. set 2ab = strain 12954, and set 4ab = strain C.

From a number of experiments with more than 100 strains selected at random and 11 »factor sera«, 5 »factor sera« were chosen (II, III, V, VI, IX) which met the following requirements:

1. High percentage of agreement between two determinations.
2. Not too many weak positive or doubtful negative reactions.
3. Strains corresponding to the immunising and absorbent antigen dried ad modum *Flosdorf-Mudd*, as this method of storing was considered at that period superior to storage in semi-fluid agar covered by liquid paraffin.
4. No (or only slight) anti-complementary tendency. In addition, it seemed expedient to employ factor sera with differing, but definite, percentages of positivity.

Using 5 factor sera, there are $2^5 = 32$ possible types if the factors are combined freely. Actually, 27 »types« were found — 9 of them with only one representative. The »types« could be established only with some difficulty, small deviations being left out of consideration.

The strains examined included 88 »sets of strains« and only 66 % of these were identical within each set of strains. This was a strikingly low percentage of identity. In particular, factor sera V and IX showed relatively wide deviations.

Comparison between the results obtained with factor determination and with cross-absorption experiments for 17 sets of strains showed fairly good agreement between the two methods. However, two sets

were found to differ after the factor serum method, while they were alike after the absorption method.

3. *Introductory Experiments with Guinea-pig Sera.*

Although, as mentioned, the anti-complementary tendency of absorbed serum could be abolished — completely or partially — by preliminary treatment of the absorbent antigen with inactivated guinea-pig serum, some other method was desirable for this purpose, because, among other reasons, the above-mentioned method was too laborious.

Another important fact was that numerous »normal« rabbit sera reacted with gonococcus antigen. Therefore, only animals with a negative gono-reaction were used for immunisation. However, this did not ensure against the formation of »non-specific« antibodies during the process of immunisation.

If these »non-specific« antibodies only corresponded to the antigen common to all gonococci, they would not play any rôle. However, this was a question about which nothing was yet known. As already mentioned, subsequent experiments showed that the antibody found in »normal« rabbit sera corresponded to the antigen which is common to all gonococci.

It seemed obvious then to try immunisation of guinea-pigs instead of rabbits. It was further found that the former very seldom gave a »spontaneous« gono-reaction.

Some preliminary experiments showed that good »remaining reactions« could be obtained after absorption of undiluted guinea-pig sera, as a rule without an anti-complementary tendency. However, later on also anti-complementary sera were encountered occasionally after this treatment.

The technique of injection was as follows: —

The guinea-pig was »heated« in an electric heating box (»mouse box«) in order to obtain congestion of the ear veins. Then Xylol was applied to the ear veins, this being also to promote congestion. Finally, the animal's head was immobilised by means of a special holder — of the same kind as that used for rabbits.

An assistant then placed and secured the guinea-pig on the lid of the heating box, so that the washed ear was illuminated from below by one of the bulbs in the box, through a hole in the lid. The injection was performed with a bevelled, reinforced hypodermic needle (No. 20). After a little training, it soon became almost as easy to intravenously inject large selected guinea-pigs (weighing at least 400 g.) as to inject rabbits.

As a rule the guinea-pigs were given 7 to 8 injections over a period of 4 weeks, i. e. about 7 cc. (7000 million bacteria). As nearly all the animals in one set had anaphylactic shock after the fourth injection, on the 11th to 12th day, and as the corresponding sera were practically all anti-complementary (0.30 % haemolysis), different preliminary

treatment of the immunising and absorbent antigen was adopted. There is probably some connection between these findings and the anti-complementary tendency.

Subsequently, as suggested by Lentz & Schäfer (1936, 1937) and by Uroma (1943), the immunising and absorbent antigen was washed with buffer saline pH 7.38 (with addition of 0.5 % formalin), carefully avoiding any admixture of culture medium. The density of the suspension was about 20000 million bacteria per cc. After incubation at 37° for 3 hours, the suspension was centrifuged, and the sediment was re-suspended in the same volume of fresh buffer saline with formalin. This was not a particularly thorough washing, but from now on none of the animals had anaphylactic shock. Altogether about 300 guinea-pigs were immunised in this way.

Here it should be mentioned, however, that among nearly 250 rabbits previously immunised with «unwashed» antigen, phenomena that might be interpreted as anaphylaxis were seen in only 3 of the animals.

Comparison of Results of Cross-absorption Experiments with 6 (7) Strains of Rabbit Sera and Guinea-pig Sera respectively.

This comparison showed some agreement between the two series of experiments, but *more partial antigens* had to be set up when using guinea-pig sera than when using rabbit sera. As a matter of fact, however, this was not very strange, since the cross-absorption experiments with rabbit sera had been carried out by examination of the antigens in fours, whereas all the 7 strains corresponding to guinea-pig sera were crossed at once. Only 3 of the 19 (72) absorbed sera turned out to be anti-complementary. In comparison, it may be mentioned that in cross-absorption experiments with the corresponding rabbit sera, an anti-complementary tendency was observed in nearly all combinations.

Factor determination with rabbit sera and with guinea-pig sera on a number of strains did not give absolutely uniform results, but the antigens used for the production of the factor sera still gave fairly uniform reactions by the two methods.

As it seemed reasonable to expect that the use of guinea-pig sera instead of rabbit sera would cause *less trouble on account of anti-complementary tendencies*, and as normal guinea-pig sera practically never reacted with mixed gonococcus antigen, immunisation of guinea-pigs was then adopted exclusively. Indeed, subsequent experiments showed that absorption of guinea-pig sera resulted only seldom in sera showing anti-complementary tendencies. Unfortunately, no direct comparison was practicable between the anti-complementary tendency in guinea-pig and rabbit sera, because, among other reasons, the same portions of antigen had not been examined systematically with both kinds of serum simultaneously.

4. *Further Experiments with Strains which, according to their Origin, might be expected to be Serologically Identical (Guinea-pig Sera).*

A number of experiments were now carried out with guinea-pig sera in the same way as those performed with rabbit sera.

a. *Factor Determination.*

Factor determination was performed on several series of urethral-cervical strains from the same patient, first with 5 factor sera, later with only 4 factor sera. Out of 76 factor-determined (5 sera) pairs of strains, 67 pairs (= 88 %) were found to be identical. Determination with factor serum II was given up subsequently, because it proved difficult to produce a sufficiently good serum, the remaining reaction gradually becoming too weak (see also the following paper).

In addition, factor determination was made on 75 strains (including 28 urethral-cervical strains) from 61 patients, corresponding to 30 chain infections.

There was fairly good agreement between the »types« within the individual chain infections, as 28 chain infections consisted of identical — or nearly identical — strains, and only 2 chain infections showed divergence of »types«.

Altogether 275 strains were examined, among which 5 »types« were particularly frequent, viz.:

Factor Formula:				
Factor serum no.: III	V	VI	IX	No. of strains
—	—	—	—	35 (13 %)
+	—	—	—	23 (8 %)
—	—	—	+	41 (15 %)
+	—	+	—	87 (32 %)
—	+	—	+	52 (19 %)

One particularly frequent »type« was found in about one-third of the cases, namely: $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} \\ + & - & + & - \end{matrix}$.

The »types« obtained were not very clearly defined, as the fixing capacity of the individual strains varied considerably within these »types«.

As to the relative frequency of the »types« obtained, most previous investigators who were able to carry out any »typing« at all agree that 1 or 2 types are particularly frequent. Thus Gordon (1921), according to a private communication to Tulloch (1922), is said to have found that 25 out of 30 strains examined could be classified into one group: »Gordon's prevalent strain«. Hermanies (1921) divided the gonococci into 6 types, among which he found 2 »main types«, namely: type I (48 %) and type II (41 %). With some difficulties, Torrey & Buckell (1922) were able to set up a »regular group«, comprising about one-half of the strains examined; and Tulloch (1922) found that 72 % of

his strains belonged to type I (Tulloch's predominant strain), a smaller number of 4 other types, and 8 unplaced strains.

Among the 3 »pure« types set up by Vollmond (1923), 2 were particularly frequent, namely: type II (41 %) and type III (50 %).

Casper (1937) divided the gonococci into 2 types: I and II. Type I was found in about 36 % of the cases and type II in about 14 %, whereas the remaining strains were distributed over a mixed type I + II, »heterologous« strains and »non-classifiable« strains (about 41 %).

Finally, by means of a specific polysaccharide, Uroma (1943) found 4 serological gonococcus types: A, B, C and D; most of the strains belonged to type A (60 %), then came type B (23 %).

It is regrettable that it has not been possible to compare some of the above-mentioned »types« with the types found in this country, but it is to be hoped that such comparison may prove practicable in the future.

b. Cross-absorption Experiments.

Cross-absorption experiments with the strains obtained in 15 chain infections showed strikingly few deviations, and the differences observed did not suggest that the respective patients did not belong epidemiologically to the same group, but could be taken to signify a certain variability of the gonococci. For instance, strains from one chain infection were found to have the same antigenic formula, thus belonging to the same »type«, but yet they were not able completely to empty each other's sera; both strains contained a partial antigen that was lacking in the other strain. Subsequent or cross-absorption experiments with several other strains showed the 2 strains in the chain infection to be very closely-related serologically.

In these experiments, anti-complementary tendency was observed only in the absorbed sera corresponding to 3 chain infections (20 %). It therefore appears as if guinea-pig sera contain less anti-complementary tendency than rabbit sera (anti-complementary tendency in 60 %). Unfortunately, however, the anti-complementary tendency in the two categories is not directly comparable, as in some cases the absorption dose chose during absorption of guinea-pig sera was as low as possible as a preliminary experiment. Furthermore, the same antigen preparation had not been examined systematically with both sera simultaneously.

Clinical examination of 61 patients corresponding to 30 chain infections offered no explanation of the deviations observed within the individual chain infections in the cross-absorption experiments and factor determinations.

The *serological differences* found within the chain infections might be explained in at least two ways: either as due to mixed infection with other gonococcus strains in the patients concerned, or resulting

from some change in the strains from *true mutation* or from a *changing of phase*. Here »true mutation« is employed for an irreversible change, »changing of phase« for a reversible change.

In all 30 chain infections, it was most likely that the strain belonged to the same type, judging by the amnestic data available, even though in many of the cases one had to consider the possibility of mixed infection, which indeed can never be excluded with certainty. Therefore, *mutation seemed to be the most probable explanation*. Demonstration of mutation in vitro, preferably from a one-cell culture, would have supported this theory. Even though this could not be done successfully, the theory was not disproved, because the conditions in vitro and in vivo were quite different. Perhaps it might have been possible in animal experiments, for instance, with blocking of the reticulo-endothelial system by means of mucin.

It had previously been decided not to use one-cell cultures because it was very difficult to obtain growth from a single gonococcus. Therefore, *single colony cultures* were employed. Unfortunately, as already mentioned, the results were negative.

A priori it seemed conceivable that mutation might arise especially in patients with *complicated or chronic gonorrhea*, or on *particular localisation of the infection* (cf. Atkin, 1925). With regard to the former possibility, out of 15 chain infections examined through cross-absorption, 6 cases were found in which the female partner perhaps had complicated or chronic gonorrhea — e. g. salpingitis — shortly before the present gonorrhea was diagnosed, or whose vita ante acta was suggestive of gonorrheal infection; 2 of these showed differences in the cross-absorption results, whereas in 3 of the remaining 9 chain infections neither partner had complicated or chronic gonorrhea. This gives the same distribution for the group with identical strains as for the group with different strains (2 out of 6 as against 3 out of 9).

As to the *significance of the localisation* to the appearance of mutations, it may be mentioned that the urethral and cervical strains may differ in the factor determination as well as in the cross-absorption test, but no rules can be laid down as to which of the two strains is likely to differ from the strain isolated from the partner. However, the material here examined was too small to elucidate this question. In addition, the conditions were complicated by the fact that it was not known which of the strains was the original. As already mentioned, some different urethral-cervical pairs behaved in a way practically similar to 7 other strains and sera, which goes against the chance of a mixed infection being the cause of the difference.

5. Cross-absorption within the »Types« found by Factor Determination.

Since a certain number of »types« could now be set up by means of factor sera, it seemed of interest to investigate to what extent the

strains within the given »types« might be related. At any rate, some deviations seemed likely, as it was not to be expected that the factor sera available would represent all the partial antigens that might occur. The following »types« were examined by means of cross-absorption experiments:

Frequency in %	Factor serum No.	Factor formula				No. of strains	No. of patients
		III	V	VI	IX		
ca. 13		—	—	—	—	10	8
» 8		+	—	—	—	4	4
» 32		+	—	+	—	10	8
» 19		—	+	—	+	7	6
Not examined		+	—	—	+	4	2

In addition, some strains that were difficult to type were examined in relation to the »types« to which they came nearest. Altogether about 300 absorptions were performed.

As an example of these cross-absorption experiments, it will be appropriate here to give the results obtained with 4 strains of »type« III V VI IX. These strains originated from 4 different patients (see also Table 4 and the survey below).

Strain No.	Sex	Origin	Pl. No.	Factor formula expressed in degrees of potency			
				III	V	VI	IX
39781 U.	F.	Chain infection	5	5	0	0	0
40914 U.	M.		6		0	0	0
42089 C.	F.		9	5½	0	0	0
41842 U.	M.		10		0	0	0

4° = 16 absorptions were performed.

Previously strain 39781 had been found to differ from 40914, while 42089 was identical with 41842. Further, the strains of the two chain infections differed with at least two antigens. The behaviour of strains 39781 and 40914 resembled that of 42089 and 41842, having thus certain points in common in spite of the difference demonstrated in the cross-absorption experiments.

The conclusion from these experiments was that in *cross-absorption experiments with strains belonging to the same »type«, determined by means of factor sera, these were found to differ unless they originated from the same patients or were part of the same chain infection.*

The antigenic differences within »type« $\begin{smallmatrix} \text{III} & \text{V} & \text{VI} & \text{IX} \\ - & + & - & + \end{smallmatrix}$ do not appear to be as great as within the other »types«.

6. Cross-absorption Experiments with Strains of different »Types« and Experiments with New Factor Sera.

It would have been interesting to compare the antigenic structure

Table 4.

Cross-absorption Experiments with 4 strains of Type α + $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} \\ - & - & - & - \end{matrix}$

Non-absorbed serum titrated in 5 tubes, absorbed sera in 2 tubes.

NB.: Undiluted guinea-pig serum, 0.025 cc. in 1st tube.

Sera absorbed on Dec. 28-29, 1943; corresponding complement fixation tests were performed on Dec. 30, 1943.

		Pt. No. 5 39781	Pt. No. 6 40914	Pt. No. 9 42089	Pt. No. 10 41842	Serum control
Non-abs. serum	39781	14	9	10	9½	0
(Pt. No. 5)						
abs. with	{ 39781 (Pt. No. 5)	0	0	0	0	0
	{ 40914 (Pt. No. 6)	5	0	0?	1	0?
	{ 42089 (Pt. No. 9)	≡ 6	≡ 6	0	0	0
	{ 41842 (Pt. No. 10)	≡ 6	≡ 6	0	0	0
Non-abs. serum	40914	12	12	8½	7½	0
(Pt. No. 6)						
abs. with	{ 39781	1	6	0?	0?	0?
	{ 40914	4	2½	4½	3½	3½
	{ 42089	≡ 6	≡ 6	0	0	0
	{ 41842	≡ 6	≡ 6	0	0	0
Non-abs. serum	42089	9	10	14	14	0
(Pt. No. 9)						
abs. with	{ 39781	0	0	≡ 6	≡ 6	0
	{ 40914	1	0	≡ 6	≡ 6	0
	{ 42089	0?	0?	0?	0?	0?
	{ 41842	0?	0	0?	0?	0
Non-abs. serum	41842	9	9	15	15	0
(Pt. No. 10)						
abs. with	{ 39781	0	0	≡ 6	≡ 6	0
	{ 40914	0	0	≡ 6	≡ 6	0
	{ 42089	0	0	0	0	0
	{ 41842	0	0	0	0	0

of the strains previously examined in cross-absorption experiments with rabbit sera, with the antigenic structure in some of the new strains that were examined with guinea-pig sera.

It had been hoped that this improved technique (employment of guinea-pig sera instead of rabbit sera) would have eliminated some of the minor differences and doubtful reactions that occurred in the preliminary experiments.

As a matter of fact, this was the case to some extent, but it has to be admitted that in cross-absorption experiments with 7 antigens using

guinea-pig sera, it was necessary to set up 9 partial antigens in order to explain the reactions observed. It was further found, however, that in cross-absorption experiments with strains of the same factor formula, these would usually prove different. Unfortunately, at this juncture (January 1944), the sera and absorption antigens were no longer available — not at all, as far as the »old« strains were concerned, and only to a very limited extent with regard to the »new« strains. Therefore, the above-mentioned comparison would have required the production of new (fresh) sera and antigens, and this could be done because, owing to the war, it was out of the question to obtain a sufficient amount of agar.

Attempts were therefore made to map out the antigenic formulas for a number of the »new« strains, taking into consideration their reactions with the 4 factor sera (III, V, VI and IX) as several cross-absorption experiments with strains and sera of differing »types« were to be carried out.

Further, strains belonging to the same sets but showing deviations in cross-absorption experiments with other strains of different »type«, were further tested in order to see whether these deviations would also appear in relation to strains which differed serologically and as regards origin.

One of the first experiments is recorded in Table 5, illustrating how 5 strains and sera were examined, and a survey of this is given below.

Survey of Table 5.

Strain No.	Pl. No.	Factor formula				Antigenic formula		Designation of antibodies in factor formula corresponding to antigenic formula II
		III	V	VI	IX	I	II	
43464	17	+	—	+	—	ab	ab ₁ b ₂	B ₁ — B ₂ —
45689	24	—	+	—	+	ae ₁	ae ₁ c ₂	— C ₂ — C ₁
46269	27	—	—	—	—	a(c ₁ c ₂)	a(c ₁ c ₂)	— — — —
44341	21	—	—	+	+	ac ₁ d	ab ₂ c ₁	— — B ₂ C ₁
40914	6	+	—	—	—	ac	ab ₁ e	B ₁ — — —

It is evident from Table 5 that strains 45689 and 46269 were able to empty each other's serum. Indeed, these sera showed quite uniform behaviour on absorption with the remaining 3 antigens — in spite of the apparently big difference in their factor formulas (see below). Antigen 44341 was also able to completely empty serum 45689, but not serum 46269.

In some subsequent experiments, the factor formulas for the 2 strains approached each other, since both strains reacted only with factor serum IX. There is thus a good deal of evidence in favour of the fact that the two strains are very closely-related.

By setting up the sera and antigens pairwise, in the way given on page 57 *ibid.*, it was possible, with certain reservation, to set up the antigenic formula I. In the case of strain 46269, the character e₁e₂ is placed

Table 5.

Cross-absorption Experiments with 5 Strains of Different Types.

Experiment performed on Jan. 11, 1944.

Sera absorbed on Jan. 10—11, 1944.

0.25 cc. undiluted serum in 1st tube.

NB.: Guinea-pig sera.

Non-absorbed sera titrated in 5 tubes, absorbed sera in 2 tubes.

		Antigen					Serum-control
		43464 Pt. No. 17 ab ₁ b ₂	45689 Pt. No. 24 ac ₁ (c ₂)	46269 Pt. No. 27 a (c ₁ c ₂)	44341 Pt. No. 21 ab ₂ c ₁	40914 Pt. No. 6 ab ₁ e	
Non-abs. serum 43464, AB ₁ B ₂ (Pt. No. 17)	Antibody content	9	6	5	6	6	0
abs. with 43464 (Pt. No. 17)		0	0	0	0	0	0
45689 (— 24) B ₁ B ₂	≧ 6	0	0	0	0□	2	0
46269 (— 27) B ₁ B ₂	5	0	0	0	0□	0□	0
44341 (— 21) B ₁	≧ 6	0	0	0	0	2	0
40914 (— 6) B ₂	6	0	0	0	0	0	0
Non-abs. serum 45689, AC ₁ C ₂ (Pt. No. 24)	Antibody content	8	10	9	8½	5½	0
abs. with 43464	C ₁ C ₂	0	≧ 6	1½	2	0	0
45689	0	0	0	0	0	0	0
46269	0	0	0	0	0	0	0
44341	C ₂	0	0□	0□	0	0	0
40914	C ₁ C ₂	0?	≧ 6	≧ 6	≧ 6	0	0
Non-abs. serum 46269, AC ₁ C ₂ (Pt. No. 27)	Antibody content	8½	9	10	8½	6	0
abs. with 43464	C ₁ C ₂	0	5	4	2½	0	0
45689	0	0	0	0?	0	0	0
46269	0	0	0	0	0	0	0
44341	C ₂	0	0□	4	0	0	0
40914	C ₁ C ₂	0	≧ 6	≧ 6	5	0	0
Non-abs. serum 44341, AB ₂ C ₁ (Pt. No. 21)	Antibody content	9	9	9	10	6	0
abs. with 43464	C ₁	0	5	0□	≧ 6	0	0
45689	B ₂	0□	0	0	≧ 6	0	0
46269	B ₂	0□	0	0	≧ 6	0	0
44341	0	0	0	0	0	0	0
40914	B ₂ C ₁	0□	≧ 6	5	≧ 6	0	0
Non-abs. serum 40914, AB ₁ E (Pt. No. 6)	Antibody content	8	6	5½	6	11	0
abs. with 43464	E	0	0	0	0	≧ 6	0
45689	EB ₁	0□	0	0	0	≧ 6	0
46269	EB ₁	0□	0	0	0	≧ 6	0
44341	EB ₁	0□	0	0	0	≧ 6	0
40914	0	1	1	1	1	2	1½

in parenthesis, thus signifying that these partial antigens are not always present — at any rate, not always in an avid form. The corresponding antibodies are designated by means of capitals, the antigens by means of small letters.

The symbol b_1 and b_2 are used instead of $b - c$, and c_1 and c_2 instead of $d - e$, in order to indicate that these partial antigens are coupled to some degree. The frequency of strains with the factor formulas $\begin{smallmatrix} \text{III} & \text{V} & \text{VI} & \text{IX} \\ + & - & + & - \end{smallmatrix}$ and $\begin{smallmatrix} \text{III} & \text{V} & \text{VI} & \text{IX} \\ - & + & - & + \end{smallmatrix}$ is about 33 % and 20 % respectively.

However, the subsequent testing of antigenic formula I proved that most of the antigenic formulas agreed, but not all of them (see also Table 5). Efforts were then made to designate the reaction of the individual strains with factor sera by means of the corresponding antibody designations. Thus, for instance, we might assume that the reaction of factor serum III with antigen 40914 is due to antibodies E and B, as this strain — in addition to the common antigen a — must also possess an antigen quota e that is different from b . This is only true, however, if we interpret the reaction with factor serum III as signifying a fixation with e as well as with b , but this cannot be taken for granted. It is most probable that antibody quota E is not represented at all in the four factor sera.

When testing formula abe for strain 40914, however, the antigenic and antibody quotas are found to be not in harmony — which again may be interpreted as indicating that antigen 40914 reacts only with part of antibody B. This assumption is also supported by the fact that the reaction of antigen 40914 with factor serum III amounts only about 3 degrees of potency, whereas a number of other antigens show 6 to 9 degrees in their fixation with factor serum III.

Nor would it be correct to take the formula of antigen 44341 as abc_1 instead of ac_1d — as one might perhaps be tempted to do in view of the factor formula.

If, on the other hand, we take the formula of antigen 43464 as ab_1b_2 , not ab_1 , so that b_1 corresponds to factor serum III — and b_2 to factor serum VI, then we may designate antigen 44341 as ab_2c_1 (instead of abc_1) and antigen 40914 as ab_1c . On testing sera and antigens pairwise, these antigen formulas are found to be in harmony; and they are called antigenic formula II.

On considering the size of the »remaining« reaction, we find no definite connection between the reaction we would expect judging by the antigenic formulas and the reaction observed. It will further be noticed from Table 5 that there are several places where the given formulas are not fitting, as corresponding reactions are »missing«. These »missing« reactions are recorded by means of the symbol \square . In particular, b_1 and b_2 (or B_1 and B_2) show »missing« reactions, but c_1 and c_2 may also fail.

It should be pointed out that it would not be reasonable to expect

the factor formulas to agree exactly with the antigenic formulas, as, for one thing, we have to assume that factor sera consist of several antibody quotas, since the absorption is carried out with only one strain per serum.

Experiments were then carried out in which 2 more gonococcus strains and corresponding sera were »crossed« with the 5 strains first examined. The result was that cross-absorption experiments with 7 strains gave 6 antigen quotas besides the common antigen a ; and it became even worse when still more strains were »crossed«.

Five additional strains were examined with cross-absorption experiments. All the factor formulas and antigenic formulas for the strains mentioned in this series are recorded in the following survey. The partial antigens with which the reaction is occasionally »missing« are put in parenthesis.

Strain No.	Patient No.	Factor formula				Antigen formula
		III	V	VI	IX	
43464	17	B_1 +	—	B_2 +	—	$a(b_1b_2)$
43467	15	B_1 +	—?	B_2 +	—	$a(b_1b_2)d$
39781	5	B_1 +	—	—	—	$a(h_1)ef$
40914	6	B_1 +	—	—	—	$a(b_1)eg$
41842	10	B_1 +	—	—	—	$a(b_1)h$
45689	24	—	C_2 +	—	C_1 +	$a(c_1c_2)$
46269	27	—	—	—	—	$a(c_1c_2)$
42981	12	—	—?	—?	C_1 +	$a(c_1c_2)$
42604	14	—	—	—	—?	$a(c_1c_2)$
30778	2	B_1 (+)	—	B_2 (+)	—	$a(a_1c_2)$
44341	21	—	—	B_2 +	C_1 +	$a(b_1c_1)i$
45046	22	—	—	B_2 +	C_1 +	$a(b_1c_1)$

In addition to the common, a antigen and some partial antigens common to some strains, several strains contained also a »special« antigen. — e. g. 43467, 39781, 40914, 41842, 44341 — while other strains — especially those with formula $a(c_1c_2)$ — appear to contain only very little »special« antigen. This is strongly reminiscent of the theory set forth by Vollmond (1923): that in addition to the *species* antigen (common to all gonococci), there are also both *type* antigens and *strain* antigens.

Conclusion:

The cross-absorption experiments carried out with 12 strains showed that, with the technique here used, it is only practicable to set up *preliminary and rather uncertain antigenic formulas*.

Further, in addition to the antigen common to all gonococci, these bacteria possess many partial antigens — perhaps both »type« antigens and »strain« antigens.

Some strains which, according to their origin, ought to be identical, while in cross-absorption experiments they turned out to be different, were probably nevertheless closely-related, as in cross-experiments with other strains they behaved similarly.

A fair degree of harmony could be demonstrated between factor formulas and antigenic formulas — except that some antibody quotas were not represented in the factor sera.

As some antibody quotas did not appear to be represented in the factor sera, an attempt was made to produce some *additional »new« factor sera*.

Experiments were made with 3 »new« sera produced by immunisation of 6 guinea-pigs per strain.

Two of these sera, however, were found to react exactly like factor sera III and IX respectively, whereas the third serum actually represented something new. From 1st April, 1944, the last-mentioned serum was used as a fifth factor serum (factor serum XII).

Summary.

An account is given of a number of cross-absorption experiments with strains which, according to their origin, might be expected to be serologically identical. These first experiments were carried out with rabbit sera. Out of 18 series of strains examined without preliminary treatment of the absorbent antigen, one or often more sera turned out to be anti-complementary, corresponding to 11 series. With the technique here used, it is not practicable to show that strains isolated from the same patient (within the same period of illness) are always identical. Experiments were then carried out with »factor sera«, i. e. particularly selected absorbed rabbit sera.

More than 100 strains picked out at random were examined with 9 »factor sera«, yielding 18 »types« with more than one representative of each and 34 »types« with only one strain of each kind — that is, a total of 52 »types«. By limiting the number of factors to 5 particularly suitable ones, 27 »types« are obtained, including 18 with more than one representative of each.

Among 88 series of strains that might be expected to be identical,

only 66 % were found to be identical when examined, using 5 factor sera.

Comparison of the results obtained from factor determination and from cross-absorption experiments carried out on 17 strains of series shows fairly good agreement between the two methods. However, 2 sets of strains are found to differ using the factor serum method, while they turn out alike in the cross-absorption experiments. Experiments with single colony antigens furnish no evidence in support of the presence of mutation or changing of phase.

Guinea-pig sera were then tried instead of rabbit sera, because, among other reasons, guinea-pigs seldom give a positive gono-reaction. The technique of injection is described. Comparison is made between the results of cross-absorption experiments with rabbit sera and guinea-pig respectively, showing a certain agreement. Factor determination with rabbit sera and guinea-pig sera respectively gave fairly uniform results.

Guinea-pigs were then used for the production of immune sera, because, among other reasons, it was thought that this would probably reduce the troublesome anti-complementary tendency.

A number of experiments similar to those performed with rabbit sera were carried out with guinea-pig sera. By factor determination on 114 pairs of strains isolated from the urethra and cervix of the same patient, 11 different types were obtained ($2^4 = 16$ possible), 5 of which appeared frequently, representing 85 % of the strains. One type was particularly frequent, being found in about 30 % of the strains. Of the 114 pairs, 103 (= 90 %) were serologically identical.

Factor determination on the strains contained in 30 chain infections showed agreement within the individual chain infections in 28 cases. Cross-absorption experiments with strains from 15 chain infections showed surprisingly few deviations, and the sera turned out to be anti-complementary only in 20 % of the cases as against 60 % when using rabbit sera. Altogether 275 strains were examined in this series.

Cross-absorption experiments with strains of the same »type« showed them to differ serologically in that they did not originate from the same patient or constitute parts of the same chain infection.

Cross-absorption with strains of different types only led to the establishment of preliminary and rather uncertain antigenic formulas. As previously found when using rabbit sera, gonococci possess many partial antigens — perhaps both type and strain antigens — in addition to the antigen common to all gonococci.

A fair degree of harmony is found between the factor formulas and the antigenic formulas.

Three »new« factor sera were tried out, one of which behaved almost like factor serum III, another almost like factor serum IX, whereas the third one appeared to contain something hitherto unknown. In subsequent studies this serum will be used as factor serum XII.

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SEROLOGICAL STUDIES ON GONOCOCCI. III.

THERMOSTABILITY AND BIO-CHEMICAL NATURE OF THE GONOCOCCUS ANTIGENS. DURABILITY OF THE FACTOR SERA. DISCUSSION.

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1. Previous Investigations.

After Heidelberger & Avery (1923), Avery & Heidelberger (1923—1925) and Avery, Heidelberger & Goebel (1925) had led the typing of bacteria into new paths by their demonstration of typespecific polysaccharides in pneumococcal capsules, similar investigations into other bacterial species were soon reported — e. g. meningococci and gonococci. Zinsser & Parker (1923) also presented some fundamental results from their work on protein-free bacterial extracts. Furthermore, Ferry & Fisher (1924) found that apparently protein-free gonococcal antigens possessed antigenic characteristics, i. e. reacting with immune sera.

Casper (1930) isolated *specific polysaccharide* and *nucleo-protein* from gonococci, corresponding to two different agglutinative types. The polysaccharide produced specific skin reactions in gonorrhoeics, whereas the nucleo-protein also produced positive skin reactions in normal subjects. Subsequently Casper (1937) reported his studies on *specific polysaccharides* carried out in 1927—1933, emphasising that to obtain good results it was essential to use freshly-isolated strains. He further stated that the specific substance corresponding to type I was *thermostabile*, being able, among other things, to stand boiling for 5 minutes, while the specific substance corresponding to type II was possibly thermolabile.

Zozaya & Wood (1932) obtained polysaccharide and nucleo-protein

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from Gram-negative cocci, including meningococci and gonococci, but only the meningococci were examined with regard to type-specificity. The authors found neither the polysaccharide nor the nucleo-protein to be type-specific.

Several other investigators have prepared a protein-free substance from gonococci, which they have used for a diagnostic skin test (Muttermilch & Grimberg (1935), Dmitriew & Demidova (1935), Barbellion & Feld (1936), Pinetti (1937). Rossett (1939) made an amorphous powder from a 4—6 day polyvalent broth culture. This powder showed properties greatly resembling polysaccharide. In contrast to previous investigators, Rossett omitted boiling of the culture, as he assumed that such treatment would destroy Casper's thermolabile polysaccharide; on intracutaneous injection of the powder he obtained specific reactions. In 1931—1934, Boor & Miller examined nucleo-protein and polysaccharide («the alcohol-precipitable fraction») in meningococci and gonococci, but they did not succeed in preparing any type-specific polysaccharide from either meningococci or gonococci.

On the other hand, in 1933—1935, Rake & Scherp succeeded in isolating specific polysaccharide from meningococci types I and II; a type III strain which they had had in stock for some time could not be distinguished from type I, and type IV could not be obtained at all at the time of these studies. The authors emphasised that there was a great difference between freshly-isolated and older laboratory strains, morphologically as well as biologically. In this connection, it may be mentioned that Torrey (1940) examined the fixing capacity of four different gonococcal antigens (Cohn (1925), Price (1932), McNeill (1932) and Kreuger (1933)) and found, by complement-fixation experiments, that the Cohn and Price antigens were about equally potent and more potent than the other two antigens. He found no conformity between the carbo-hydrate content (Molisch-test) and the complement-fixing capacity, from which he concluded that the carbo-hydrate content played no rôle in the complement-fixation.

In France (Boivin and associates (1933)), a «gluco-lipoid» has also been obtained from various Gram-negative bacteria, in addition to polysaccharide and nucleo-protein, which are not always chemically well-defined substances. This «gluco-lipoid» substance, which is antigenic, can only be prepared from Gram-negative and not from Gram-positive bacteria. Chev   (1933) prepared type-specific «gluco-lipoid» corresponding to three meningococcus types, and Boor & Miller (1939) prepared an antigenic substance from meningococci and gonococci ad modum Boivin. Subsequently, in 1944, the latter authors described in detail the procedure for the preparation of «gluco-lipoid», in addition to a number of experimental studies of the antigenic properties of this preparation. The authors arrived at the conclusion that the «gluco-lipoid» was only relatively specific as regards gonococci and

meningococci. However, after hydrolysis, during which the carbohydrate was separated from the lipoid, the former gave a specific precipitation, making it possible to distinguish gonococci from meningococci, and meningococci of type I from meningococci of type III.

Menzel & Rake (1942) continued Rake & Sherr's immuno-chemical studies on meningococci. They isolated a new kind of type-specific substance, which they called »Kappa« and which they assumed differed from the ordinary type-specific polysaccharides (pneumococci) and from type-specific proteins, such as, for instance, Lancefield's (1928) M-substance and Verwey's (1940) staphylococcal protein.

»Kappa« was not destroyed by proteolytic enzymes, and consisted probably of protein with an enzyme-resistant prosthetic group which contained polypeptides and some carbo-hydrates.

This substance was prepared from a 2—3 weeks old broth culture, to which was added 0.5 % Phenol and which was centrifuged in Sharples' super-centrifuge (30,000 r. p. m.). The supernatant fluid was filtered in various ways, and then the filtrate was concentrated by ultra-filtration. The protein was precipitated at pH 4.5, and from this precipitate the crude »Kappa« substance was prepared by tryptic digestion.

In 1943, Uroma described a method for typing gonococci by means of a specific polysaccharide prepared ad modum Casper.

Complement-fixation was used for serological examination. Uroma was able, by examination of 30 strains with four sera, to divide the gonococci into four types, of which three-fifths of the strains were of the same type. Uroma prepared only four sera, and thus did not investigate whether sera produced with different strains within the same type were strictly type-specific. The purification of the polysaccharides was rather difficult, as at least six solutions and precipitation with alcohol acid and basic milieu alternately were required in order to avoid anti-complementary tendencies in the antigen.

In experiments on immunisation of rabbits with the *purified polysaccharides*, no immunity was obtained. In addition, the peculiar fact was observed, that the *same polysaccharides* which gave positive complement-fixation gave absolutely negative precipitation reactions.

In 1943, Stokinger, Ackerman & Carpenter reported the results of micro-chemical analysis of gonococcus strains (four fresh and two old) grown in Douglas broth. Among other things, they found carbohydrate to constitute from 5 % to 9 % of the weight of dried bacteria, lipoid 10—14 % and nucleo-protein 60—65 %. They found two nucleo-protein fractions: a relatively insoluble lipo-nucleo-protein, and a lipoid-free nucleo-protein. Furthermore a lipoid was isolated which was found to be not particularly characteristic of gonococci.

It was not practicable by chemical methods to demonstrate the presence of a type-specific polysaccharide in gonococci grown in Douglas broth. On the other hand, in the broth from gonococcus cultures, they found a *protein-like fraction* with toxic and antigenic pro-

perties. This purified fraction reacted with gonococci as well as with other Gram-negative cocci in complement-fixation tests.

Stokinger, Carpenter & Plaek (1944) investigated the immunological relationship in *Neisseria* by means of quantitative agglutination ad modum Heidelberger & Kabat. They found no tendency to group or type formation in material consisting of nine gonococcus strains. Meningococci and gonococci were more closely related than the gonococci were to each other. However, the gonococcus strains examined differed in that strains showing equally strong cross-reactions as a rule gave rise to immune sera differing in antibody components.

Certain gonococcal elements proved to be soluble in normal rabbit serum with physiological pH values, and the last-mentioned authors assert that this property has not been observed in bacteria whose carbohydrate is the most important factor in their antigenic specificity. As an illustration, it may be mentioned that meningococci of group I (assumed to contain specific carbo-hydrate) were insoluble in normal rabbit serum at physiological pH, whereas meningococci of group II (assumed to contain specific protein) were soluble to about the same degree as gonococci. It is not possible to see from this paper whether these experiments were carried out with the same or different normal rabbit sera. This point may be of some interest, as the solubility conceivably signify an antigen-antibody reaction in serum depending upon the amount of antibody aimed against bacteria serologically related to gonococci, e. g. *Pasteurella*, *Micrococcus catarrhalis*.

2. Writer's Investigations.

2. Occurrence of Capsules.

It is only natural to associate the occurrence of specific polysaccharides in bacteria with the presence of a capsule in the organisms concerned, as in several bacterial species the capsule has been shown to contain just the type-specific polysaccharide, e. g. pneumococcus.

Generally, however, the gonococci are considered to be non-capsular (cf. Thomas & Bayne-Jones (1936)), though there are some authors who think they have found capsulated gonococci, e. g. Israeli (1921), Szilvasi (1932), Almaden (1938) and Bernstein (1941). It is, however, difficult to exclude the possibility of the phenomena thus observed being artificial products from fixation or staining, or being due to some optical phenomenon.

With regard to the occurrence of capsules in gonococci, a single observation has been carried out with a living gonococcus culture suspended in physiological saline solution with addition of methylene blue. The culture suspension was examined under the microscope as follows:

- 1) Without any addition;
- 2) With addition of normal rabbit serum, gono-reaction negative;

- 3) With addition of normal human serum, gono-reaction negative;
- 4) With addition of homologous rabbit serum, gono-reaction strongly positive.

On addition of homologous rabbit serum, a somewhat dubious swelling of the bacterial body observed, but no actual capsular swelling. Something similar could be seen in a moist unstained smear.

b. Thermostability.

In order to investigate whether genuine and absorbed sera (factor sera) represented antihodies against thermolabile or thermostable antigens (of protein or of polysaccharide-like lipid character respectively), complement-fixation tests were made with gonococcus antigens treated in various ways. An example of this is given in Table 6.

Table 6.
Factor Determination on 2 Gonococcus Strains of Different Types.

Studies carried out with antigen prepared from »living« gonococci, and on gonococci heated to different temperatures during various periods. In addition, the experiment is carried out with non-absorbed sera corresponding to the two strains.

The experiment was performed on 7th March, 1945.

Sera absorbed on 5th & 6th March, 1945. 0.025 cc. undiluted serum in the first tube.

Figures = degrees of potency.

Antigen 12954	Guinea-pig factor sera					non-absorbed Guinea-pig sera	
	III	V	VI	IX	XII	12954	2c
»Living« culture	9	0	8½	0	0	11½	7
Heated for 20 min. to 56°	9	0	8½	0	0	11½	6½
» » » » » 37°	8½	0	7	0	0	10	8½
» » » » » 100°	9	0	8	0	0	11½	8½
» » 40 » » »	9	0	7½	0	0	12	7
» » 1½ hrs. » »	9	0	7	0	0	11	6
» » 3 » » »	9	0	8½	0	0	12	6
» » 6 » » »	9	0	6½	0	0	12	6
<i>Antigen 2c</i>							
»Living« culture	0	0?	0	8½	0	5½	11½
Heated for 20 min. to 56°	0	0?	0	8	0	4½	11½
» » » » » 37°	0	0	0	6½	0	4½	11½
» » » » » 100°	0	0	0	0?	0	4½	9
» » 40 » » »	0	0	0	0	0	5	9
» » 1½ hrs. » »	0	0	0	0?	0	3½	9
» » 3 » » »	0	0	0	0	0	3½	6
» » 6 » » »	0	0	0	0	0	2½	6

Antigens corresponding to two strains of different »type« (12954 and 2c) were used untreated — i. e. »living cultures« suspended in saline, density of 1000 millions per cc. — and heated for 20 minutes

to 37° and 56° respectively, and also for varying lengths of time to 100° on water-bath.

Five factor sera (III, V, VI, IX, XII) were examined, together with the non-absorbed immune sera corresponding to the two strains. (Reyn, Alice; *ibid.*).

The fixing capacity of strains 12954 and 2 c differed in their reaction to heating. Strain 12954 reacted similarly both with factor sera and with non-absorbed serum (homologous and heterologous) with all antigenic portions; 2 c lost its fixing capacity for factor serum IX on heating to 100° for 20 minutes, while there was no difference with »living« gonococci, whether heated to 37° or 56°. The reaction with non-absorbed sera was also reduced, though to a lesser degree (2—5 degrees of potency), fairly proportionately to the time.

In these cases, fixing with the non-absorbed sera revealed the difference in the antigenic structure of the two strains, the reaction being considerably stronger with homologous sera than with heterologous sera (*cf.* Casper's »specific« sera corresponding to types I and II. (1930, 1937)).

These experiments support the assumption of a difference in the character of strains of »types III—IV« and »types V—IX«. In order to corroborate this view, an experiment was carried out in which ten strains were heated to 56° for 20 minutes and to 100° for one hour. Six of these strains (four of which reacted with factor serum IX, one with factor serum V and one with factor serum XII), lost their fixing capacity for the factors mentioned by being heated to 100° for one hour, whereas the fixing capacity of four strains of »type III—IV« or »type III« remained unchanged. In two or three strains of »type V—IX«, the fixing capacity for homologous sera was lowered, while it remained unchanged in the rest.

To sum up these findings, it may be stated that the fixing capacity for non-absorbed (homologous and heterologous) serum is affected only to a slight degree by heating the antigen to 100°, as compared to heating to 56°. This might indicate that the antigenic quota common to all gonococci is chiefly of carbo-hydrate, or possibly lipoid, character.

As it seemed more likely that variations in the fixing capacity would become more conspicuous if too low a dose of the antigen was used, the same ten antigens and one new antigen were tested with four non-absorbed sera in the same way as above. In four tests where the homologous serum was used, the fixation with »100° antigen« was unchanged or accentuated. The fixing capacity for heterologous sera was *unchanged or stronger* in nine antigens, and weaker in two, one of which reacted with factor serum II alone, the other with factor serum IX alone. The experiment thus confirms that the antigen common to all gonococci is thermostabile.

In some cases, the reactions with 100° antigen were stronger than

»Kappa« substance in meningococci agreed fairly well with the results of experiments with gonococci heated to 100° dealt with in the present work. In both cases the results indicated that substances of protein character might be type-specific.

The experiments reported by Miller and associates (1943) dealing with heating of gonococci suspended in saline, may be correlated with the results of our *complement-fixation experiments with non-absorbed gonococcus sera* tested with antigens heated to 56° and 100° respectively. Miller *et al.* found, amongst other things, that when meningococci are heated to 80° for more than four hours they become agglutinable with non-type-specific meningococcus sera as well as with heterologous immune sera.

Conclusion.

No capsular swelling is observed on the addition of homologous rabbit serum to stained and unstained smears of gonococcus cultures.

There are thermostabile as well as thermolabile gonococcus antigens; the antigen common to all gonococci is thermostabile and, therefore, probably of carbo-hydrate character (perhaps lipid).

The partial antigens corresponding to factor sera III and IV are mainly thermostabile (polysaccharide, or possibly lipid?), whereas the antigens reacting with factor sera V and IX are mainly thermolabile (proteins?).

In some cases, fixation with antigen heated to 100° is stronger than with antigen heated to 56°. This may perhaps be explained by thermolabile antigen inhibiting the fixation with thermostabile antigen.

The results obtained so far, however, are to be regarded merely as preliminary, and the writer fully realises that more thorough investigations into this particular field ought to be carried out.

c. Experiments on Preparation of Polysaccharide.

Attempts have been made to produce polysaccharide ad modum Uroma, firstly from strain 12954, of »type« $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} & \text{XII} \\ + & - & + & - & - \end{matrix}$ and later also from strain 2 c, »type« $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} & \text{XII} \\ - & - & - & + & - \end{matrix}$.

The procedure was as follows: —

An 18-hour ascites agar plate culture was washed once with saline. Then four volumes 10 % sodium taurocholate was added, and the mixture was shaken thoroughly for 30 minutes in a shaking machine, heated on water-bath at 56° for 30 minutes, shaken for 30 minutes, and left standing for 24 hours at room temperature. Next day it was heated on water-bath for 30 minutes at 56°, precipitated with normal acetic acid, allowed to stand in ice-box for 24 hours, and centrifuged. The sediment was discarded and the supernatant fluid precipitated with 96 % alcohol while being stirred. Even this first precipitation requires a large amount of alcohol (5 volumes), but on addition of quarter volume ether, a considerably smaller amount of alcohol will

suffice. Uroma merely states that he precipitates with 95 % alcohol, and that more alcohol has to be used for each precipitation. According to later information, it is understood that acetone was used for the precipitation.

After centrifuging, the scanty white sediment was dissolved in distilled water, and the solution rendered basic with 0.1 normal NaOH. Alcohol and ether were added, and the mixture left over night in refrigerator. Next day, centrifuging and solution of the sediment in distilled water was carried out. Normal acetic acid was added and precipitation with a larger amount of alcohol and ether carried out, then the mixture was allowed to stand overnight in refrigerator, and so on, as a rule with four precipitations in acid and basic milieu alternately. Crude polysaccharide (precipitated and dissolved only once) was found to have anti-complementary tendencies — as reported by Uroma — but after four precipitations and solutions, these tendencies disappeared. The fixing capacity was very weak in proportion of the relatively large number of gonococci used for the preparation of this substance (about 200 large plates yielded 4 cc. polysaccharide solution, and 0.05—0.10 cc. of the solution is used for each tube. As mentioned above, the purified »polysaccharide« in moist form dissolved easily in water and the solution gave a negative biuret reaction and negative Fehling's reaction that turned positive after acid hydrolysis.

Experiments have been carried out with two preparations (P. 2c and P. 12954) obtained from two strains of different types (tested with the corresponding gonococcus immune sera). The results obtained are recorded in Table 7.

Table 7.

Two polysaccharide preparations made from gonococcus strains 2c and 12954 (P. 2c and P. 12954) with saline suspensions heated to 56°, tested by complement-fixation experiments with corresponding immune sera.

Figures = degrees of potency.
Experiment carried out August 1946.

Guinea-pig sera		P. 2c		P. 12954		2c, 56°		12954, 56°
	No. 1	2½		0		9		8
	» 2	0		0		9		9
Immun-	» 3	½	aver.	0	aver.	Not examined		
ised	» 4	3	1,1	1½	0,3	»	»	
with 2c	» 5	0		0		»	»	
	» 6	1½		0		»	»	
	No. 7	2½		2½		8		9
	» 8	5		1		7½		9
Immun-	» 9	3	aver.	0	aver.	Not examined		
ised	» 10	5½	3,3	0	0,6	»	»	
with 2c	» 11	2½		0		»	»	
	» 12	2½		1		»	»	

It is evident from Table 7 that the two polysaccharide preparations are not type-specific, since for example, P. 2 c has a stronger fixation than P. 12954 with serum produced by immunisation with strain 12954. The sera are produced through immunisation with a simple suspension of gonococci in saline. In order to obtain the maximal reaction with the polysaccharide antigens, it was necessary to use a rather high dose, viz. 0.10 cc. per tube, as 0.01, 0.02 and 0.04 cc. gave only a weaker fixation, or no reaction at all. In this connection, it should be mentioned that maximal fixation with gonococci suspended in saline was obtained with a dose of about 5×10^7 million gonococci per test tube. For the preparation of 0.10 cc. polysaccharide solution, at least 5×10^{10} gonococci, or 1000 times more, were used, and even then the fixation obtained was not so strong, viz. $2\frac{1}{2}$ —5 degrees of potency, as against $7\frac{1}{2}$ of potency.

Conclusion.

It seems reasonable to assume that the polysaccharide thus obtained corresponds to the antigen common to all gonococci, which, as mentioned above, is thermostabile. It was perhaps not to be expected that a type-specific polysaccharide might be obtained from strain 2 c, as the type-specific antigens of this strain were *thermolabile* and thus, presumably, of protein character. Unfortunately, technical conditions for the preparation of this polysaccharide have made it impossible to continue these experiments for a time, but the intention is to try to obtain polysaccharides from strains of varying types whose antigens are thermostabile.

d. Durability of the Factor Sera.

Complement-fixation experiments with the antigens which react with factor sera III and IV gave practically constant reactions with the factor sera, irrespective of whether the factor sera were specially prepared or about two weeks old. On the other hand, it gradually became more evident that the results with antigens reacting with factor sera V and IX were highly variable. Presumably this was due to weakening of the factor sera in the few days during which they were stored at $+4^\circ$. (Experiments with storage at -8° often resulted in gelatinisation of the sera when thawing.)

Portions of factor sera V and IX were prepared, in addition to antigens from strain 1 a (\approx Type \llcorner

III	V	VI	IX	XII
—	+	—	—	—

) and 2 c (\approx Type \llcorner

III	V	VI	IX	XII
—	+	—	+	—

). The potency of the factor sera was tested against the same dose of antigen the same day, the next day and the day after. On this occasion, antigen 20 reacted with factor serum V and with factor serum IX, while as a rule 2 c reacted only with factor serum IX. As early as the day after preparation of the serum, i. e. having been centrifuged after absorption overnight, both factor sera

showed a reduction in potency by 3—5 degrees of potency, and on the following day this was even slightly more pronounced.

On account of these findings, it is necessary to use freshly-absorbed sera corresponding to factor sera V and IX and probably also factor serum XII, whereas factor sera III and VI may be kept for months.

This experiment also shows that the antigens giving rise to fixation with factor sera V and IX are destroyed by heating to 100°.

In order to make sure that *attenuation of the antigens* might not have been the cause of the apparently decreasing potency of the sera, fresh factor sera were tested partly with recently-prepared antigen, and partly with antigen prepared 1, 2, 3 and 30 days before. However, no impairment of the reactions could be demonstrated in this way.

As mentioned before, it was not realised at the beginning that the varying reactions with factor sera IX and V were due to weakening of the serum — because, among other reasons, all the factor serum was, as a rule, used up in one experiment, and, furthermore, the experiment was performed on the same day as the serum was prepared. A review of the earlier results shows very many deviations as far as factor sera V and, particularly IX, are concerned.

Perhaps the presence of *type-specific antibodies corresponding to both protein and carbo-hydrate* may explain the findings of Torrey (1940), in which an apparently carbo-hydratefree antigen reacted type-specifically. Another interesting thing concerning factor sera is the question of the constancy or variability of the reactions obtained with the same antigens tested with different factor serum preparations.

During the period October 1941 to March 1944, a total of 12 antigens were tested frequently with the factor sera available at different times. These antigens were chiefly obtained from strains corresponding to the immunising and absorbing antigens in the production of factor sera. The antigens were examined with a varying number of portions of serum. Unfortunately, it was impossible to establish afterwards the age of the antigens at the time of the individual tests. Furthermore, it could not be stated with complete certainty which serum (rabbit number, etc.) had been used in every instance. Most of the antigens were examined 20 to 30 times, and none less than ten times.

It was possible to ascertain, by means of repeated examinations, whether »anticipated« (usually homologous) reactions appeared again, and, if this is the case, to observe how other (probably less well-known) antigens behaved, i. e. whether or not their reactions with the factor sera are constant.

For illustration of this, it will be appropriate to give details for some of the factor sera.

Factor serum II (immunising antigen 12937, absorbing antigen 11423 (now discontinued):

At first, the serum gave good homologous reactions, even though it

often had some anti-complementary tendencies. The serum failed both in homologous and heterologous fixation on going on to preliminary treatment of the absorbing antigen with guinea-pig serum. As with the guinea-pig sera, the first set (5 animals) gave good potent sera, but in the next two sets (each consisting of 6 animals), only two guinea-pigs gave a usable serum, in spite of the fact that freshly-prepared antigen was used for the third set. It may be that the antigenic formula for strain 12937 had undergone some change, as might be indicated also by the increasing tendency to fixation with factor serum III. As mentioned previously, employment of factor serum II was discontinued in October 1943.

Factor serum V (immunising antigen 1 a, absorbing antigen 12954):

As already mentioned, the reaction with this serum was dependent on whether or not the serum was freshly-absorbed. This applied to homologous as well as to heterologous fixations. It is a rather strange fact that strain 11394 reacted with this serum as long as rabbit serum was used, but not when using guinea-pig serum.

It seems conceivable that in this case also a change may have taken place in the antigenic structure, either corresponding to the immunising strain (1 a), the absorbing (12954) or strain 11394.

These experiments and many others showed that often factor sera III and VI, and V and IX respectively, were »coupled«, i. e. that a strain reacted either with III and VI, and not with V and IX, or the opposite.

However, factor serum III, for example, was by no means identical with factor serum VI. There were many strains which reacted with factor serum III alone (nearly to the titre limit), while the reverse was very rare.

It was known from various earlier experiments that strains 12954 (= immunising antigen corresponding to factor serum III) was closely related to strain 11413 (= immunising antigen corresponding to factor serum VI). With regard to this, it should be mentioned that the sera were absorbed by two strains differing in factor formula, viz. 1 a and 11394 respectively.

Nor were factor sera V and IX identical, as some strains reacted only with one of them, and cross-absorption experiments showed that the strains used for immunisation differed with a least two partial antigens.

Conclusion.

Factor sera V, XII and IX cannot be kept satisfactorily by storage at 4°, whereas factor sera III and VI may keep for several months. It is therefore important to use freshly-absorbed sera corresponding to factor sera V, IX and XII.

Consequently the results of some of the earlier cross-absorption

experiments must be accepted with a certain degree of reservation. Strangely enough, the antigens which gave fixations with factor sera V and IX were thermolabile. Tests carried out with different factor serum preparations and the same antigens showed that most of the »anticipated« reactions were to be found again. However, there are various indications that at any rate some gonococcus strains may change in regard to antigen. Factor sera III and VI, as well as factor sera V and IX, are coupled to some extent without being identical.

Discussion.

To try to assemble the experiences gained with regard to the antigenic structure of gonococci, we arrive at the following results:

As a rule, absorption is required for serological differentiation of the gonococcus strains. However, for reasons unknown, a few immune sera (but only a few) are so »specific« that certain serological differences can directly be demonstrated (e.g. in complement-fixation experiments). Probably these conditions are due to individual differences in the experimental animals. Possibly it was such accidental occurrence of »specific« sera which caused Casper (1930, 1937) to directly classify gonococci into types I and II.

There is a thermostable antigen that is common to all gonococci, in addition to several partial thermolabile and thermostable antigens. The antigenic structure is independent of the degree of drug resistance.

The question of the occurrence of a type-specific polysaccharide (Casper, Uroma) has only been touched upon in the present work and cannot yet be settled. However, there is much to indicate that, as well as this possible type-specific polysaccharide, the gonococci also contain a type-specific thermolabile protein substance (cf. Menzel & Rake (1945), and Stokinger, Ackerman & Carpenter (1943)).

By means of four to five »selected« factor sera, i. e. absorbed sera, the antibody contents of which are only partially known, the gonococci may be divided into a number of serological »types«, one of which is particularly frequent, while four are relatively frequent.

Cross-absorption experiments within the individual »type« show that strains of the same kind usually differ serologically unless they originate from the same patient, etc.

In cross-absorption experiments it very seldom happens that strains originating from different sources are found to be identical.

Therefore, in addition to the common gonococcus antigen and one or more »type-specific« partial antigens demonstrable by means of factor sera, there are one or more »strain-specific« partial antigens (cf. Vollmond's (1923) theory on species antigen, type antigen and strain antigen).

It seems most probable that, by means of the factor sera (which

were partly chosen at random), we have found the more common partial antigens or sets of partial antigens.

The antigens corresponding to factor sera III and VI and to factor sera V and IX are further partly coupled, so that reaction with sera III and VI as a rule excludes reaction with V and IX. Thus, in several ways, we are able to differentiate between the two kinds of antigen and sera.

For one thing, factor sera V and IX (XII) are different from factor sera III and VI with regard to durability, the former becoming attenuated quite quickly on storage at $+4^{\circ}$, while the latter keep for several weeks under the same storage conditions.

Furthermore, the antigens corresponding to reaction with factor sera V and IX (perhaps XII also) are thermolabile, in contrast to the antigens corresponding to factor sera III and VI.

Some experiments indicate that gonococcal strains may change in their antigenic composition with partial antigens either appearing or disappearing (possibly only quantitatively, cf. Hermanies (1921). However, this does not appear to happen very often.

In experiments with single-colony antigens from the same strain in several »generations«, no antigenic changes or differences could be demonstrated by means of factor sera.

It may be that the method of storage has some influence on the antigenic composition, but it would be difficult to demonstrate this with certainty.

If we use the »typing« made possible by the use of factor sera, we will not always obtain the same »type« on examination of strains which, according to their original, ought to be identical, e. g. simultaneous cultures of urethral and cervical strains from the same patient. This may conceivably be due to mixed infection in the patient, or may result from mutation. As mentioned, no antigenic differences could be demonstrated in the single-colony experiments.

Whether this is a question of mutation or mixed infection is very difficult to decide. Perhaps the drug or chemo-resistance might be associated with the serological type. If in some patients the urethral and cervical strains are found to be distinctly different, in serological as well as drug resistant aspects, it seems most probable that mixed infection is the cause. However, this requires an accurate method for determination of the drug resistance, unless the two strains happen to show very different drug resistance.

By using cross-absorption experiments to control the points of difference and resemblance observed, fairly good agreement between the factor serum diagnosis and the cross-absorption results can be found, but it is a striking fact that some sets of strains are found to be quite different by factor determination and identical by cross-absorption.

One may be more certain that the two strains concerned are

actually different when this is indicated both by factor determination and cross-absorption experiments.

Thus, if we fairly often find strains which, according to their origina, ought to be identical, while serologically they turn out to be different, this will naturally detract from the serviceability of »typing« by means of the available factor sera for use in epidemiological (or medicolegal) investigations. It may, therefore, be concluded that, in its present form, the »typing« is not particularly suitable for this purpose.

It is not practicable to decide with certainty whether or not two strains are identical, except through production of immune serum with consequent cross-absorption experiments. However, it has never been observed so far that a strain of factor formula $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} & \text{XII} \\ + & - & + & - & - \end{matrix}$ proved to be identical on cross-absorption with the strain of the formula $\begin{matrix} \text{III} & \text{V} & \text{VI} & \text{IX} & \text{XII} \\ + & - & + & - & - \end{matrix}$.

With regard to the utilisation of these results in the production of antigen for the diagnostic gono-reaction, experiments will have to show whether it is practicable, by the use of a few strains picked out on the basis of the reaction with factor sera, to obtain an equally — or even more — specific and sensitive antigen than the one available in the present polyvalent, more accidentally composed, diagnostic antigen.

Summary.

A fairly detailed historical survey is given of previous investigations into the bio-chemical nature of gonococcus antigens, showing that this question is far from settled yet.

Then follows an account of the writer's investigations which, among other things, indicates that the antigen common to all gonococci is thermostabile. In addition, there are both thermostabile partial antigens (corresponding to factor sera III and VI) and thermolabile partial antigens corresponding to factor sera V and IX. In some cases the antigenic fixing capacity proved to be stronger when the antigen was heated to 100° than when heated to 56°, possibly because a thermolabile antigen may inhibit the fixation of a thermostabile antigen.

It has not been possible to demonstrate any capsular swelling on addition of homologous rabbit serum to stained and unstained bacterial smears.

Factor sera V, IX and XII could not be kept satisfactorily when stored at +4°, whereas factor sera III and VI may keep for months at that temperature. Experiments with storage of factor sera in refrigerator often resulted in gelatinisation.

Furthermore, it appears as though the strains may undergo changes

in their antigenic structure on storage and cultural transfer in the laboratory.

Finally, the writer discusses the experiences gained with regard to the antigenic structure of the gonococci, and deals with all the results obtained, including those reported in the two preceding papers.

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STUDIES ON THE MODE OF ORIGIN OF PENICILLIN- RESISTANT STAPHYLOCOCCI*)

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Previously it was the prevailing idea that acquired resistance to antibacterial agents arises through a process of adaptation. Now it is more commonly assumed that acquired resistance arises through selection in this way that any fairly large population of a sensitive bacterial strain will contain a few resistant variants brought about by mutation — independently of the presence of the antibacterial agent concerned — that will be able to grow when the sensitive individuals in the culture have been eliminated (*Dubos*⁷), *Luria*¹³)).

A few authors have contested the correctness of the latter theory, however, asserting that it must be a matter of adaptation even though they also emphasize more or less ardently the significance of a concurrent selection (*Hinshelwood*¹²), *Abraham, Callow & Gilliver*¹)), *Bellamy & Klimek*²) assume that penicillin-resistant strains of *Staphylococcus aureus* arise through mutations, but at the same time they think that these strains probably do not appear spontaneously but are induced by penicillin.

As to acquired penicillin resistance, the theory about spontaneous mutations is based upon works reported by *Demerec*^{1, 5}). On spreading a staphylococcus culture on penicillin-containing agar he found that a certain portion of the bacteria survived and formed colonies all of which at the highest penicillin concentration (0.064 O. U. per cc.) were found to be more resistant than the original strain, whereas some colonies at lower concentrations showed a normal sensitivity to penicillin. On transfer to agar containing 0.064 O. U. per cc., 30 samp-

*) Supported by a grant from the Carlsberg Foundation.

les from independent cultures of the staphylococcus strain were found to vary greatly in the number of resistant colonies, while the number of colonies resulting from transfer of 20 samples of the same culture turned out to be constant. On the basis of this experiment *Demerec* concluded that the resistant bacteria have appeared in the original broth culture through a spontaneous mutation and then on transfer to penicillin agar they have grown out in colonies, all the sensitive bacteria being eliminated. For, if it were a change in the sensitivity induced by penicillin, it would be reasonable to assume that the number of resistant colonies even from independent cultures would keep fairly constant. According to *Demerec*, the great variations in the number of resistant bacteria from different cultures can be explained only through the occurrence of spontaneous mutations. When only a few resistant colonies appear, the mutation has taken place late in the growth of the culture; when many resistant colonies turn up, on the other hand, the mutation has taken place early, so that a large number of resistant bacteria could be formed through division of the mutants.

This theory has been supported by many investigators. Here it will suffice to mention merely a work of *Spink & Ferris*¹⁴). These authors first state that there are two different forms of penicillin-resistant staphylococci: 1) strains rendered resistant in vitro and characterized by inability to form penicillinase, and by their resistance being lost on transfer to media free from penicillin; and 2) staphylococci that have become resistant in vivo and, like the primarily resistant staphylococcus strains occurring in nature, are capable of forming penicillinase, and their resistance is stable.

Demerec mentions that 10 of his penicillin-resistant strains had shown no loss of resistance after 20 transfers in penicillin-free broth. Accordingly, *Spink & Ferris* set up these resistant strains as a contrast to the strains described by other authors which were rendered resistant by cultivation in liquid penicillin-containing medium. For, as already mentioned, the latter strains are characterized by their resistance being unstable. Even though *Demerec* says nothing about the capacity of his strains for penicillinase production, *Spink & Ferris* then classify them together with the persistent penicillinase-producing strain that may be isolated after treatment with penicillin, and they set up this stable hereditary resistance — which, according to *Demerec*, thus arises through spontaneous mutation — as a contrast to the unstable resistance which, in their opinion, arises through adaptation.

Spink & Ferris find additional support for their view in some experiments in which some penicillinase-producing staphylococcus strains were transferred frequently in ordinary penicillin-free broth and showed an increase in resistance. This the authors think, has to be explained in this way: such a culture — just as described by *De-*

merec as applying to ordinary non-penicillinase-producing strains — will include a few bacteria possessing a greater resistance, and through the frequent transfers these bacteria have a chance of increasing in number.

This explanation seems rather improbable as in these experiments with penicillin-free medium, of course, there is no possibility for the selection that plays such a decisive role in *Demerec's* theory. Undoubtedly a selection taking place merely because of the frequent transfer is out of the question; otherwise we should have to assume that the »resistant variants« through the frequent transfers obtain a higher rate of growth than the other bacteria in the culture. No doubt the explanation of the result is that after the frequent transfers the culture forms a larger amount of penicillinase simply as a result of the more vigorous growth or the more vigorous manifestations of growth resulting from the frequent transfers.

As emphasized in preceding papers,^{9, 10)} the most natural explanation of the difference between staphylococci rendered resistant *in vitro* and resistant strains isolated after treatment with penicillin will be that the latter strains merely are primarily resistant strains, which — on account of an original mixed infection, or because of a secondary infection — predominate after the sensitive bacteria have been eliminated. This explanation has been suggested also by *Demerec et al.*⁹⁾

Furthermore, from the experiments to be reported in the following, there appears to be no difference between staphylococci rendered resistant through the usual method in fluid media and after the method employed by *Demerec*, on solid medium. Presumably, then, the theories advanced by *Spink & Ferris* may be left out of consideration.

Writer's Investigations.

The experiments to be presented here were carried out with a strain of *Staphylococcus aureus* No. 70, which was employed also in experiments previously reported on the effect of penicillin on staphylococci.

When penicillin-containing broth is inoculated with sufficiently many staphylococci, in some penicillin concentrations there will be a secondary growth consisting of bacteria with greater resistance than that possessed by the original strain. In order to look further into this fact, the following experiment was carried out (Table 1):

Table 1.

Number of colonies on agar squares after spreading of one loopful from cultures of *Staphylococcus aureus* containing various concentrations of penicillin and with an initial bacterial concentration from 10^{+1} to 10^{+6} of an 24 hour's broth culture.

	0	2	4	6	8	10	12	24	72 hours
10^{+1}									
1 O. U.	∞	∞	+++	+++	+++	+	30	0	0
$1/2$ »	∞	∞	+++	+++	+++	++	34	4	0
$1/4$ »	∞	∞	+++	+++	+++	++	24	8	+++
$1/8$ »	∞	∞	+++	+++	+++	++	13	50	+++
$1/16$ »	∞	∞	+++	+++	+++	++	60	+	+++
$1/32$ »	∞	∞	∞	∞	∞	∞	+++	++	∞
10^{+2}									
1 O. U.	+++	+++	+++	+	12	0	1	0	0
$1/2$ »	+++	+++	+++	+	22	8	1	2	0
$1/4$ »	+++	+++	+++	+	16	4	3	0	+++
$1/8$ »	+++	+++	+++	+	3	0	2	75	+++
$1/16$ »	+++	+++	+++	+++	+	+	40	+++	∞
$1/32$ »	+++	+++	∞	∞	∞	+++	+++	+++	∞
10^{+3}									
1 O. U.	+++	++	+	10	4	0	1	0	0
$1/2$ »	+++	++	+	22	5	0	3	0	0
$1/4$ »	+++	++	+	24	6	1	0	0	+++
$1/8$ »	+++	+++	+	7	1	0	0	0	+++
$1/16$ »	+++	+++	++	7	1	0	1	+++	+++
$1/32$ »	+++	+++	+++	+++	+++	+++	+++	+++	+++
10^{+4}									
1 O. U.	+	16	9	1	0	0	0	0	0
$1/2$ »	+	44	8	1	1	0	0	0	0
$1/4$ »	+	38	8	2	0	1	0	0	0
$1/8$ »	+	+	9	1	0	0	0	0	0
$1/16$ »	+	+	22	0	0	0	0	∞	∞
$1/32$ »	+	+	++	++	+	+	+	∞	∞
10^{+5}									
1 O. U.	7	0	0	0	0	0	0	0	0
$1/2$ »	9	6	0	1	0	0	0	0	0
$1/4$ »	10	3	1	0	0	0	0	0	0
$1/8$ »	8	10	1	0	0	0	0	0	0
$1/16$ »	4	14	1	0	0	2	0	+++	+++
$1/32$ »	7	11	15	12	6	7	5	∞	∞
10^{+6}									
1 O. U.	0	1	0	0	0	0	0	0	0
$1/2$ »	1	0	0	0	0	0	0	0	0
$1/4$ »	2	0	0	0	0	0	0	0	0
$1/8$ »	0	0	0	0	0	0	0	0	0
$1/16$ »	0	1	0	0	0	0	0	0	0
$1/32$ »	1	1	3	5	9	9	40	∞	∞

∞ confluent growth. ++ 100—200 colonies.
 +++ nearly confluent growth. + 50—100 colonies.

A series of tubes with 4.5 cc. broth containing penicillin in different concentrations, are inoculated with 0.5 cc. of a 24-hour culture of staphylococcus 70, undiluted as well as diluted 1:10, 1:100, and so on, making the initial bacterial concentration in the tubes 10^{-1} , 10^{-2} , 10^{-3} , etc. The tubes are incubated at 37° , and at various points of time transfers are made with a platinum loop on abt. 20×20 mm. squares on agar plates.

From this experiment it is evident that, depending on the initial amount of bacteria transferred, a secondary growth will appear at certain penicillin concentrations. As mentioned, this growth consists of bacteria possessing a higher degree of resistance to penicillin than the original strain. With large inocula secondary growth is found in penicillin concentrations of up to $\frac{1}{4}$ O. U. per cc., with smaller inocula only at lower penicillin concentrations, and with an initial bacterial concentration of 10^{-3} there is no secondary growth at all. When the inocula are sufficiently small, the secondary growth often comes long after the appearance of »one loop sterility«. With larger inocula, as a rule, at the corresponding junctures a few colonies will be seen which on determination of the resistance are found to be made up of bacteria with normal sensitivity to penicillin. No doubt they are persisters as described by Bigger.³⁾ From the figures for bacterial concentrations 10^{-2} and 10^{-3} it is evident that the number of these persisters is no greater in the penicillin concentrations giving secondary growth than in the concentrations where no such growth is seen.

Even this experiment suggests strongly that the theory advanced by Demerec is not correct, and that the resistant bacteria appear only after contact with penicillin for some length of time. But, of course, with the technique here employed, where bacterial count is carried out only for a very small volume (about 0.002 cc.) it will not be justifiable to draw any definite conclusion as to the possibility of an early occurrence of a few resistant bacteria.

The significance of the bacterial inoculum to the secondary growth has been studied more thoroughly in a number of experiments where the appearance of the growth in a fluid medium was observed. The results vary somewhat from one experiment to another. As a rule a penicillin concentration of $\frac{1}{8}$ O. U. per cc. has shown growth in all the tubes where the initial bacterial concentration was 10^{-4} and also in several tubes with 10^{-5} but not in any tubes with 10^{-6} . In an experiment with 100 tubes containing 5 cc. with $\frac{1}{8}$ O. U. per cc. and an initial bacterial concentration of 10^{-5} , secondary growth was observed in 25 tubes. Only in six of these tubes could any growth be made out after 48 hours, and in no instance did growth appear within 24 hours. In the remaining tubes it took longer for the growth to appear, and in 6 tubes it appeared only after 6 days of incubation. In all the cases a secondary growth was found to consist in bacteria with increased penicillin resistance. In these cases where the secondary growth appears at such a late juncture, presumably it can be taken

for granted that the growth does not originate from resistant bacteria present in the original culture.

Other experiments have shown that when the inoculum is sufficiently large the secondary growth will appear constantly at penicillin concentrations of up to $\frac{1}{8}$ O. U. per cc., as a rule also at $\frac{1}{4}$ O. U. per cc., but only in very few instances at $\frac{1}{2}$ O. U. and never at higher concentrations. Thus the highest penicillin concentration at which secondary growth is fairly regular is $\frac{1}{4}$ O. U. per cc.; and this is also

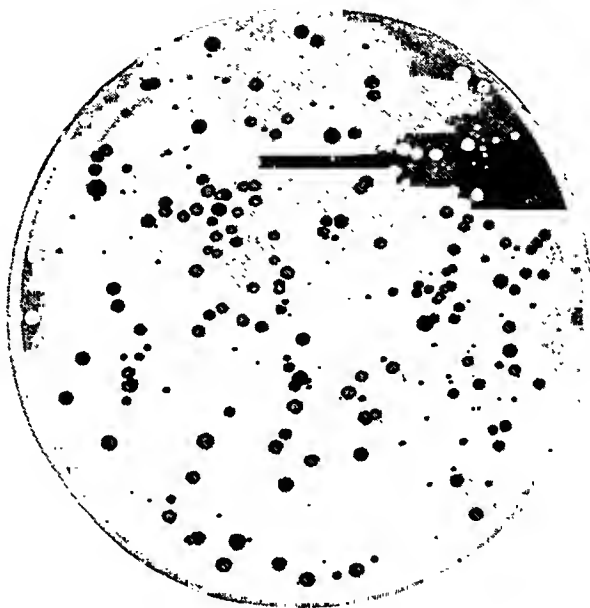


Fig. 1.

Colonies of *Staphylococcus aureus* on agar containing $\frac{1}{8}$ O. U. penicillin per cc. after 48 hour's incubation at 37° C.

the highest concentration at which the staphylococcus strain here employed will regularly show primary growth before the bactericidal and lytic action of penicillin asserts itself.⁸⁾ So it seems obvious to assume that the appearance of penicillin-resistant bacteria is dependent on such growth. In those cases where a small inoculum has been employed, and the resistant bacteria therefore have been very late in their appearance, of course, the real primary growth has ceased long ago and we have to assume that the culture now consists of the aforementioned persisters exclusively. After a long state of rest, however, such persisters may again germinate and go through exactly the same primary growth and subsequent lysis as other bacteria in the culture.⁸⁾ In this way, then, it should be possible for the persisters to form resistant bacteria in the same way as other bacteria in a penicillin-containing culture.

In addition some experiments were carried out with solid media in the same way as described by Demerec. Fig. 1 shows an agar plate

containing $\frac{1}{8}$ O. U. per cc.. On the surface, by means of an angular glass rod 0.1 cc. of a 24-hour broth culture of staphylococcus 70 is spread and the plate incubated at 37° for 48 hours. It will be noticed at once that we here meet with colonies differing greatly in size, from about 3 mm. in diameter to quite tiny, almost invisible colonies. There are quite gradual transitions between the various forms of colonies. On determination of the resistance of 48 colonies from two plates of this kind, all of them — regardless of their size — were found to consist of bacteria with increased resistance to penicillin. Even this finding appears to show that the explanation given by *Demerec*, that such colonies represent resistant variants present in the original culture, cannot be correct, as otherwise all the colonies would be expected to be of the same size. Undoubtedly the polymorphous picture of the colonies is to be explained in this way: that here we are dealing with colonies differing in age, as resistant bacteria may develop at different points of time here on a solid medium as well as in a liquid medium.

In order to look further into the development of the resistant bacteria, the following experiment was performed (Table 2): A flask containing 50 cc. broth with $\frac{1}{8}$ O. U. penicillin per cc. and an initial bacterial concentration of 10^{-1} (from a 24-hour old broth culture) was incubated at 37° and at different intervals 0.5 cc. was transferred from the flask to agar plates containing penicillin in various concentrations. After 48 hours' incubation at 37° the colonies on the plates were counted.

This experiment shows plainly that the penicillin-resistant bacteria cannot have been present in the original broth culture. For in that case we would expect for each penicillin concentration to find that the number of resistant colonies would increase gradually from the beginning of the experiment. At any rate, we would not find any fall in the number of resistant colonies, as is plainly evident in this experiment. The transitory rise in the number of colonies on agar containing $\frac{1}{8}$ O. U. per cc. seen after 1—3 hours may be explained readily as due to the higher number of bacteria present in the culture at these points of time as a result of the primary growth. In addition to the experiment here described, two quite similar experiments were performed with exactly the same result.

In his experiments, as mentioned *Demerec* found that staphylococci rendered penicillin-resistant with the method he employed would keep their resistance unchanged even after 20 passages in penicillin-free broth — in contrast to staphylococci rendered resistant in fluid medium. Thus it might look as if there were a difference in the staphylococci rendered resistant by employment of these two different methods. For this reason, the above-mentioned 48 strains isolated from agar containing $\frac{1}{8}$ O. U. per cc. — all of which showed an increased resistance to penicillin — were transferred daily on ordinary broth. After

Table 2.

Number of colonies on agar plates with various concentrations of penicillin after spreading 0.5 cc. from a culture of *Staphylococcus aureus* containing $\frac{1}{8}$ O. U. penicillin per cc.

	No. of colonies on penicillin agar	Total no. of bacteria per cc.		No. of colonies on penicillin agar	Total no. of bacteria per cc.
0 h.			8 h.		
1 O. U.	0	44×10^6	$\frac{1}{4}$ O. U.	0	9×10^4
$\frac{1}{2}$ »	0		$\frac{1}{8}$ »	5	
$\frac{1}{4}$ »	1		$\frac{1}{16}$ »	26	
$\frac{1}{8}$ »	9		$\frac{1}{32}$ »	90	
$\frac{1}{16}$ »	66				
$\frac{1}{32}$ »	∞		12 h.		
1 h.			$\frac{1}{4}$ O. U.	0	$< 10^4$
$\frac{1}{4}$ O. U.	0	82×10^6	$\frac{1}{8}$ »	0	
$\frac{1}{8}$ »	20		$\frac{1}{16}$ »	3	
$\frac{1}{16}$ »	60		$\frac{1}{32}$ »	3	
$\frac{1}{32}$ »	∞		24 h.		
2 h.			1 O. U.	0	50×10^2
$\frac{1}{4}$ O. U.	0	69×10^6	$\frac{1}{2}$ »	0	
$\frac{1}{8}$ »	14		$\frac{1}{4}$ »	0	
$\frac{1}{16}$ »	35		$\frac{1}{8}$ »	5	
$\frac{1}{32}$ »	∞		$\frac{1}{16}$ »	8	
3 h.			$\frac{1}{32}$ »	100	
$\frac{1}{4}$ O. U.	0	50×10^6	48 h.		
$\frac{1}{8}$ »	16		1 O. U.	0	10×10^4
$\frac{1}{16}$ »	32		$\frac{1}{2}$ »	0	
$\frac{1}{32}$ »	∞		$\frac{1}{4}$ »	∞	
4 h.			$\frac{1}{8}$ »	∞	
$\frac{1}{4}$ O. U.	0	9×10^6	$\frac{1}{16}$ »	∞	
$\frac{1}{8}$ »	11		$\frac{1}{32}$ »	∞	
$\frac{1}{16}$ »	24				
$\frac{1}{32}$ »	∞				

10 transfers only a few of the strains showed decrease in resistance, that is, partly confirming the result reported by *Demerec*.

But, when staphylococci that have been made resistant by growing in fluid medium lose their resistance even after a few passages in penicillin-free broth, this does not merely signify a real reversion but also — and, I think, above all — that the slowly growing resistant bacteria become overgrown by the normally sensitive bacteria present in the culture as persisters.⁹⁾ With the method here employed, where the resistant bacteria form well-defined colonies on penicillin agar, there will naturally be a greater chance of obtaining resistant bacteria in pure culture, and on subsequent transfer to penicillin-free medium only the reversion of the resistant bacteria will assert itself. This often

takes place very slowly, as is evident from experiments reported previously where staphylococcus strains rendered resistant in fluid medium yielded resistant colonies which on further cultivation gave strains whose resistance remained unchanged through prolonged subcultivation.⁹⁾

Presumably these observations will explain the difference noticed between strains that were rendered resistant on solid and fluid media, respectively.

The same 48 strains were examined for penicillinase formation by means of the satellite method described by Gots.¹¹⁾ In no instance could any penicillinase formation be demonstrated. One strain, which through repeated passages on penicillin agar had been made resistant to 2 O. U. per cc., was transferred to broth containing 2 O. U. penicillin per cc.; and after incubation at 37° for 48 hours, a Seitz filtrate of the culture showed no loss in penicillin activity.

So presumably there can be no doubt that the resistant staphylococcus strains obtained after the two different methods are of the same character.

As mentioned, *Demerec's* theory about the penicillin resistance arising from spontaneous mutations was based on experiments that showed a very wide variation in the number of resistant colonies obtained in subcultures on penicillin agar from independent cultures, whereas the number of such colonies remains constant for several subcultures from the same culture. *Demerec's* experiment was performed as follows: 0.3 cc. of a diluted culture containing about 300 bacteria per cc. was placed in each of 30 small test tubes which then were incubated at 37° for 18 hours. It was from subcultivation from these cultures on agar that *Demerec* found the wide variation in the number of resistant colonies. On the other hand, the number of samples from a single culture that on agar showed a constant number of resistant colonies were taken from an 18-hour culture containing 15 cc. of broth inoculated with 0.3 cc. of the same dilute culture as was used for the small tubes. But such a culture is not directly comparable to cultures of only 0.3 cc. inoculated with the same number of bacteria and incubated for the same length of time. It is obvious that after 18 hours' incubation the small cultures are physiologically older than the large one.

Table 3 shows the outcome of an experiment performed after the same principles as given by *Demerec*, but including also independent cultures of 15 cc. A 24-hour broth culture of staphylococcus aureus 70, containing 66×10^7 bacteria per cc. was diluted with broth to 10^{-6} , and samples of 0.3 cc. of this dilution were placed in 10 small test tubes. In addition, 10 tubes containing 15 cc. broth were each inoculated with 0.3 cc. of the same diluted culture. After incubation at 37° for 24 hours — during which the small tubes were stoppered with rubber stoppers in order to avoid evaporation — samples of 0.1 cc.

Table 3.

Number of colonies of *Staphylococcus aureus* on agar containing $\frac{1}{8}$ O. U. penicillin per cc. after spreading samples at 0,1 cc. from 10 independent cultures at 0,3 cc., 10 independent cultures at 15 cc. and 10 samples from the same culture at 15 cc.

Cultures at 0,1 cc			Cultures at 15 cc		
Culture No.	Total no. of bacteria per cc	No. of colonies on penicillin agar	Culture No.	Total no. of bacteria per cc	No. of colonies on penicillin agar
1.	102×10^7	120	1.	58×10^7	46
2.	106×10^7	ab. 400	2.		50
3.	128×10^7	60	3.		44
4.	99×10^7	235	4.		42
5.	111×10^7	94	5.		62
6.	82×10^7	45	6.		43
7.	101×10^7	38	7.		36
8.	98×10^7	124	8.		39
9.	108×10^7	175	9.		29
10.	77×10^7	ab. 300	10.	53×10^7	42
			10.		50
			10.		52
			10.		44
			10.		41
			10.		58
			10.		39
			10.		32
			10.		46
			10.		46

were transferred from each tube to an agar plate containing $\frac{1}{8}$ O. U. penicillin per cc.; from one of the larger cultures altogether 10 samples were transferred to different plates. After incubation at 37° for 48 hours, the resistant colonies were counted.

From Table 3 it will be noticed that the subcultures from the small cultures show a very wide variation in the number of resistant colonies — just as observed by *Demerec*. In contrast hereto the subcultures from the 10 large cultures showed a fairly constant number of resistant colonies, and here the variation is no greater than that found for 10 samples from the same culture.

According to this experiment, the explanation of *Demerec's* result is presumably to be found in conditions characteristic of small cultures, and thus it cannot be taken to support the theory about the resistance arising from spontaneous mutations. How this particular difference between small and large cultures is to be explained, however, is a question I am unable now to decide.

From the experiments here described, I think, there can be no doubt that we shall have to discard the theory advanced by *Demerec* and assume that penicillin-resistant staphylococci do not appear until the given culture has been in contact with penicillin for some length

of time. But whether their appearance be due to adaptation or to a mutation induced by penicillin is a question which at any rate cannot be settled through these experiments. Still, it is plainly evident that at any rate it is not a matter of adaptation in the sense that the total culture becomes accustomed to growth in a penicillin-containing medium. The experiments illustrate very well the significance of the selection which Demerec has emphasized so strongly. During their growth in a penicillin-containing medium only a few bacteria out of a given culture will become resistant to penicillin.

Summary.

The appearance of penicillin-resistant strains of *Staphylococcus aureus* under cultivation on penicillin-containing medium is investigated by employment of solid as well as fluid media.

The resistant bacteria obtained with the two different methods appear to be of quite the same character.

Secondary growth of resistant bacteria is observed only in penicillin concentrations where a primary growth takes place. On this account, then, it has to be assumed that the appearance of penicillin resistance is dependent on such growth.

The experiments also appear to show that penicillin-resistant bacteria arise only after contact with penicillin for some length of time — in contrast to the prevailing theory about spontaneous mutation.

The variation observed by Demerec in the number of resistant colonies in subcultures on penicillin agar from independent cultures could be seen only on employment of very small cultures, whereas the number of resistant colonies was constant when cultures of 15 cc. were used for this purpose. So the findings reported by Demerec cannot be taken to prove that the resistance to penicillin arises through spontaneous mutations.

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TUMOUR PRODUCTION USING POLYETHYLENE GLYCOLS AS CARRIERS FOR CARCINOGENIC HYDROCARBONS

By Kai Setälä.

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The appearance and incidence of tumours following the application of carcinogenic hydrocarbons to the skin is highly dependent on the nature of the solvent. There are, in addition, profound differences in the action of any solvent according to whether it is applied externally or by *e. g.*, subcutaneous injection. It is not known whether the solvents depend on physical and/or chemical characteristics for their effects.

Benzene, acetone, chloroform, *etc.* compounds have been the most commonly used solvents for the topical application of the carcinogens. Acetone has been preferred to benzene as a solvent for carcinogenic hydrocarbons because acetone has less effect upon the skin.¹²⁾ Acetone has also been found to have a definite accelerating effect on the action of some carcinogens when applied to the skin. This has been attributed to the water-miscible property of acetone.⁴⁾ Epidermal carcinogenesis has, in general, been obtained somewhat more rapidly with acetone than with benzene as a solvent for *e. g.*, 0.3 per cent methylcholanthrene.¹⁷⁾ The volatile solvents mentioned above are satisfactory as pure liquids with reproducible properties but applied to the skin sometimes produced untoward pathological symptoms in this organ, and in the case of benzene, caused changes in the blood picture of the host. For subcutaneous, intravenous or oral administration they are of course out of the question.

However, certain investigations, *e. g.*, experiments with such added substances which are not fat-soluble («cocarcinogenesis»),¹⁵⁾ and investigations concerning the histological localization of the carcinogens in certain phases of the nuclear cycle (living cells, tissue cultures), cannot be carried out in such a media. Attempts have, therefore, been made to circumvent the limitation of insolubility to water.

The »solvents« used for these purposes include e. g., colloidal suspensions in water or gelatin,³⁾ suspensions in serum,¹⁰⁾ glycerin,⁷⁾ aqueous solutions of soap,¹⁾ »Postonal«,¹⁶⁾ sodium desoxycholate-water solutions,¹⁰⁾ etc. *Ekwall* and *Setälä*^{5, 6)} have in other connections reported some observations concerning cutaneous tumours in mice when the skin has been painted with carcinogenic hydrocarbons dissolved in certain association colloids. It appeared that when painted the skin of mice the carcinogen (9.10-dimethyl-1.2-benzanthracene) was rapidly assimilated and by the fluorescence microscope it was accumulated in the same places as when applied by painting with acetone solutions. It appeared further that after about 13—14 weeks from the beginning of the experiment all the animals had developed tumours.

The writer has used some *polyethylene glycols* as carriers and spreaders for carcinogenic hydrocarbons.^{13, 14, 15)} It appeared that when for instance, the non-volatile »Carbowax 1500« (trade mark for certain polyethylene glycols) was used as carrier, the carcinogen presumably could permeate into the epithelial cells more easily, and further, on using dioxane (diether of glycol) instead of the above mentioned compound, the microscopical appearance seemed to be about the same as in the case of acetone. In another connection it appeared that treating the mouse skin with carcinogen dissolved in dioxane or »Carbowax 1500« resulted after certain periods of time in multiple, progressively growing warts. The writer, as far as he knows, is the only one to have used polyethylene glycols as spreaders for carcinogenic hydrocarbons in skin painting.

Material and Methods.

The group of animals used in the present work included 280 mice of both sexes of a mixed strain bred in this laboratory. The animals were divided into subgroups, 10 mice in each. The mice were carefully examined twice weekly and notes made regarding their general appearance, epilation of the skin, ulcerations, warts, etc.

The incidence of tumours was estimated (in per cent) from the number of mice surviving at the appearance of the first tumour or tumours.²⁾

As carcinogenic hydrocarbons 20-methylcholanthrene and 9.10-dimethyl-1.2-benzanthracene (Eastman Kodak Company, Rochester, N. Y.) were used.

As solvents the following compounds were used: (1) reagent grade acetone (control series), (2) »Carbowax 1500« (Carbide and Carbon Chemicals Corporation, N. Y.) and (3) dioxane (L. Light & Co., Ltd. Wraysbury, Mddx.).*) The carcinogens were dissolved in dioxane to

*) »Carbowax 1540« and »Carbowax 4000« have also been at the writer's disposal.

give a concentration of 0.03 and 0.3 per cent respectively, in acetone to give the concentration of 0.09, 0.03, 0.3 and 0.36 per cent respectively. In the case of »Carbowax« the corresponding concentrations were 0.25 and 1.0 per cent. In the case of acetone and dioxane (*i. e.*, volatile solvents) the mice received drops of the solutions on a large area of the back. In the case of »Carbowax« (*i. e.*, non-volatile solvent) the animals were painted with the unmelted compound. The mice were treated two, three or six times weekly. In the case of subcutaneous injection the carcinogen was first dissolved in »Carbowax«, and the solvent was then mixed with an equal quantity of water. Then this compound was injected subcutaneously to give a dose of about 2 mgm carcinogen in 0.5 ccm of aqueous »Carbowax«.

The whole tumours as well as certain other tissues were fixed in neutral formalin and stained with haematoxylin-van Gieson.

The Nature of Polyethylene Glycols.

The glycols are a group of aliphatic dihydric alcohols. The lower glycols are viscous, clear liquids, and the higher ones are crystalline. The non-volatile polyethylene glycols (*i. e.*, »Carbowax«) differ from lanolin in that they are readily soluble in water (*e. g.*, »Carbowax 1500«: solubility 62 per cent at 20°.¹¹) According to the literature the p_H of a 0.5 per cent aqueous solution of this compound was 4.8 as compared with 5.2 for distilled water alone. These compounds also have the property of lowering the surface tension of water, the addition of 0.1 per cent by weight of »Carbowax 1500« resulting in a solution with a surface tension of 58 dyn., and a 1 per cent solution, 38 dyn. in contrast with water — which has a surface tension of 72.8 dyn./20°.¹¹)

Dioxane (diether of glycol) is an oily liquid, which is miscible with water in all proportions. Both dioxane and »Carbowax« have been used as »detergents«.

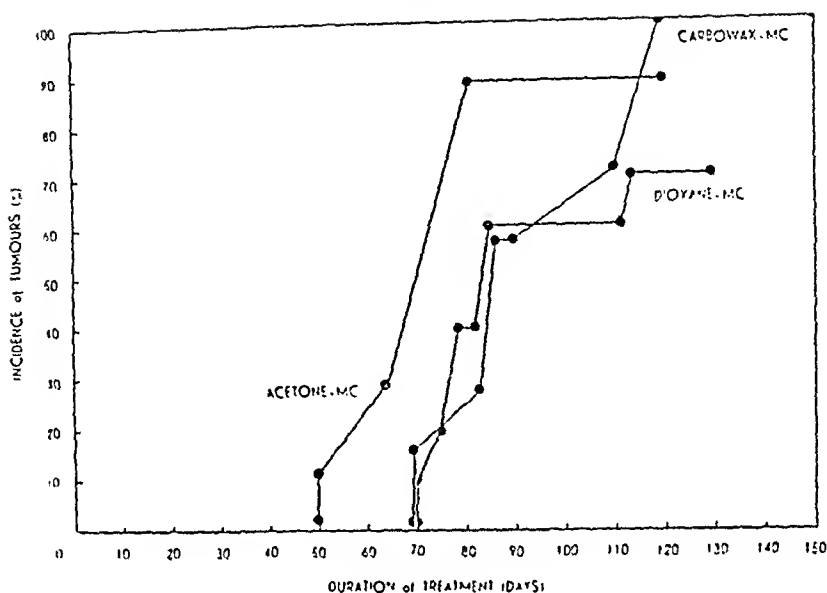
The writer has carried out some experiments treating, for instance, *Paramaeciae* with aqueous solutions of »Carbowax«. This compound was, in certain concentrations, non-toxic to these animals (unpublished data).

Incidence of tumours.

In the following paragraphs some illustrative series will be presented:

Acetone series (i. e., control series).

20-methylcholanthrene. — (Graph 1.) By the 2nd and 3rd week of the experiment about 50 per cent of the animals showed a distinct epilation (0.36 per cent of carcinogen in acetone). However, by the



Graph 1.

Incidence of cutaneous tumours produced by 20-methylcholanthrene using various solvents. The concentrations of the carcinogen: 0.36 per cent in acetone (applied 3 times weekly), 0.3 per cent in dioxane (applied 3 times weekly) and 1.0 per cent in »Carbowax 1500« (applied 6 times weekly).

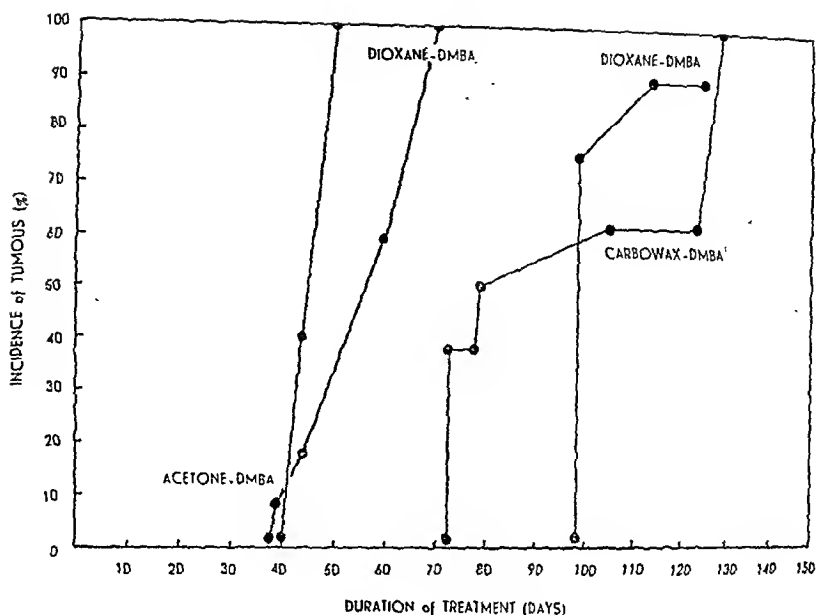
Abscissae: duration of treatment (in days).

Ordinates: incidence of tumours (in per cent).

4th week the hairs started to regrow in some of the animals, so that fewer mice showed complete epilation. The first solitary growing wart appeared by the 7th week of the experiment (*i. e.*, after about 21 applications of the carcinogen). All of the surviving animals, *i. e.*, about 90 per cent of the mice surviving at the appearance of the first wart, had multiple progressively growing warts by the 11th and 12th weeks of the experiment (34 applications). Macroscopical signs indicating malignancy were seen by the 10th week (29—30 applications). By the 16th week of the experiment there were large tumours in the treated area of the skin of mice (Fig. 1).

»The average response« in these series was 6½ weeks.

9.10-dimethyl-1.2-benzanthracene. — (Graph 2.) At the end of the 1st week of the experiment a considerable number of the animals showed some degree of epilation, and by the 5th week the skin of all the mice was epilated (0.3 per cent carcinogen in acetone). The first warts in these series appeared in the 6th week (19 applications). During the following 10 days, *i. e.*, by the 6th and 7th weeks of the experiment (20—25 applications) the incidence of tumours reached the figure 100 per cent. There was no regrowth of the hairs in the painted area in the mice. Warts appeared simultaneously at short intervals in the treated area at varying distances from each other, but



Graph 2.

Incidence of cutaneous tumours produced by 9,10-dimethyl-1,2-benzanthracene in various solvents. Concentration of the carcinogen 0.3 per cent in acetone and dioxane, and 0.25 per cent in «Carbowax 1500» (applied 3 times weekly).

they quickly became confluent and formed a single large, and often, ulcerating tumour. Symptoms indicating malignancy were seen by the 10th week of the experiment (30 applications).

»The average response« in the above mentioned series was 6 weeks.

Dioxane series.

20-methylcholanthrene. — (Graph 1.) At the end of the 2nd week of the experiment about half of the experimental animals showed some degree of epilation, but by the 5th week there were no epilated areas in the painted regions of the mice (0.3 per cent carcinogen in dioxane). A new epilation process began by the 8th week of the experiment. The first solitary growing wart appeared at the end of the 10th week (30 applications). The number of tumours increased and in the 12th week (36 applications) about 50 per cent of animals had tumours. After this time the number of takes increased slowly at the same time as the hairs began to regrow. When the experiment terminated after about 16 weeks (50 applications) the incidence of tumours had increased to 70 per cent. The painted solution was well tolerated. (Fig. 4.)

»The average response« in these series was 12 weeks.

9,10-dimethyl-1,2-benzanthracene. — (Graph 2.) The incidence of tumours varied considerably in different series. In Graph 2 two extreme curves are presented. In the first series »the average response«

was about $8\frac{1}{2}$ weeks, in other series about 14 weeks respectively. — Thus, for instance, in the later case up to the 4th and 5th weeks of the experiment the growth of the hairs had been slower, but not until by the 11th week (36 applications) were the majority of the animals epilated in the treated area. However, during the following week the hairs began to regrow. All the mice were epilated in the 13th week of the experiment. The first, partly solitary, partly multiple growing warts appeared in the 14th week (43 applications). By the 16th week (47 applications) 90 per cent of the animals, i. e., all surviving animals, had tumours. The painted solution was well tolerated.

»The average response« was 14 weeks.

»Carbowax« series.

(1) Skin paintings.

20-methylcholanthrene. — (Graph 1). — (a) 1.0 per cent carcinogen dissolved in »Carbowax 1500« applied six times weekly: The epilation process in the painted area of the back began in the 6th week of the experiment (35 applications). The epilation process was comparatively slow, so that e. g., in the 15th week there was a distinct regrowth of the hairs in all animals. The first solitary growing wart appeared in the 10th week (62 applications), then the number of tumours increased so that the incidence was 50 per cent (i. e., of all mice surviving) after 12 weeks (90 applications). The incidence was 100 per cent in the 17th week (100 applications). Symptoms indicating malignancy were observable by the 20th week of the experiment (115 applications). When the experiment terminated the majority of the animals had large, ulcerating tumours (Fig. 11).

»The average response« was 12 weeks.

(b) 0.25 per cent carcinogen dissolved in »Carbowax 1500« applied three times weekly: The epilation process began in the 9th week (26 applications). The first tumour appeared by the 18th week (54 applications).

»The average response« in these series was 21 weeks.

In both series mentioned above the mice gained in weight suggesting that the painted medium was well tolerated.

9.10-dimethyl-1.2-benzanthracene. — (Graph 2.) There was no preceding epilation, as generally seen in the experiments of this kind, in these series, but the epilation and the appearance of tumours developed simultaneously. The first warts appeared in the 10th week of the experiment (30 applications). After this the incidence of tumours increased, and 50 per cent of all animals had tumours by the 12th week (35 applications). All the mice had warts after 18 weeks from the beginning of the series. The first signs indicating

malignancy appeared by the 11th week. There were considerable differences in the size, appearance and multiplicity of tumours (Cf. Figs. 7 and 8). The painted medium was well tolerated.

»The average response« was 11 weeks:

(2) Subcutaneous injections.

20-methylcholanthrene. — The first subcutaneously situated tumours appeared by about the 14th week of the experiment. The injected media was well tolerated in general. However, in some mice there developed cutaneous necroses at the site of injection.

9.10-dimethyl-1.2-benzanthracene. — The subcutaneously situated tumours appeared at the same time as in the foregoing cases. In one case (mouse No. 10/I) there was a great, ulcerating and expansively growing tumour resembling cutaneous carcinoma macroscopically (Fig. 14). The injected medium was, in general, well tolerated.

Microscopical Appearance.

In the followings paragraphs some microscopical data on tumours induced by two carcinogenic hydrocarbons in polyethylene glycols will be given. A more detailed description will be given later on.

Acetone series (for control purpose). — The most common type of mouse tumours induced by 20-methylcholanthrene and 9.10-dimethyl-1.2-benzanthracene dissolved in acetone was the squamous cell carcinoma with cornification (Figs. 2 and 3). The tumours in the present series differed in no way from those reported in the literature. The uncornified squamous cell carcinomas and the ordinary basal cell carcinomas were not seen in these series. It was interesting to note that in the 9.10-dimethyl-1.2-benzanthracene series, at the time when the experiment terminated, there were numerous tumours showing acuminate papillae tipped with heavy masses of horny material, but no distinct invasive properties (Cf. the dioxane groups).

Dioxane series. — The developmental stage of the tumour foci, as well as the external appearance of the neoplasms varied greatly even in the same animal. There was no direct correlation between the size of the tumours and their microscopical appearance. Even in small tumours there were often highly invasive properties observable (Cf. Fig. 4, 5 and 6). In the majority of cases instead of normal keratinization the tumour cells had undergone parakeratotic changes as seen, for instance, in Fig. 5. The mitotic activity varied also in different parts of the same tumour; and in some tumours with definite morphologic characteristics of malignant proliferation there were no mitotic figures.

observable. In general, the tumours in these series were identical in all morphological respects to those seen in experimental carcinogenesis.

»Carbowax« series. — The group of mice treated with the carcinogens mentioned dissolved in the non-volatile »Carbowax« showed a considerable incidence of tumours, both innocent and malignant. The microscopical appearance did not differ from that seen in the foregoing series. Thus the neoplastic tissue has very often undergone parakeratotic changes (Fig. 12). There were often large areas with numerous mitoses (Fig. 13). As in the case of dioxane there was no direct correlation between the size of the tumours and the histological malignancy (Cf. Figs. 11, 12, 13 and 8, 9 and 10).

In the cases of *subcutaneous injections* the treatment resulted in subcutaneously growing sarcomas (Fig. 16). Sometimes, as seen in Fig. 14, the injected material could be seen to reflow through the injection channel. The treatment at times resulted in tumours consisting of either neoplastic tissue of epidermal origin or of more complex material infrequently associated with hyperplastic or sarcomatous proliferation of the elements of mesenchymatous origin of the cutis. Thus e. g., in mouse No. 10/I there was squamous cell carcinoma with cornification (Fig. 15), and fibro-sarcoma.

Conclusions.

The experiments above demonstrate that both volatile (dioxane) and non-volatile (»Carbowax 1500«) polyethylene glycols are capable of acting as solvents and carriers for 20-methylcholanthrene and 9.10-dimethyl-1.2-benzanthracene in skin carcinogenesis in mice. When comparing the effectiveness of the solvents mentioned above, it appears that the epilation process is somewhat faster in the acetone series, but varied considerably in the dioxane series. It was very slow in the »Carbowax« series. Both in the dioxane and »Carbowax« experiments there is, sometimes, a distinct regrowth of the hairs after the primary epilation wave. Sometimes no epilation of the painted area was seen at all. »The average response« (the 50 per cent incidence of tumour-bearing animals estimated from the number of mice surviving at the appearance of the first tumour or tumours) in the dioxane series varied greatly. Sometimes it was nearly the same, sometimes, however, twice longer than that in the acetone series. In the »Carbowax« series it was also longer than in the acetone experiments.

The evidence presented is satisfactory proof that the epidermis of the mouse is capable of producing a great number of tumours, both innocent and malignant, when certain polyethylene glycols are used as spreaders for the carcinogenic hydrocarbons in question. The neoplasms produced by carcinogens dissolved in these compounds differ microscopically in no way from those seen in skin carcinogenesis in general.

»Carbowax 1500« is a solvent about equally suitable both for skin paintings (in the melted or unmelted state) and for subcutaneous injections (in weak aqueous solutions).

The polyethylene glycols mentioned above have a particular characteristic: they are readily soluble in water. This is a very important quality, for instance, in studies concerning cocarcinogenic phenomena.¹⁵⁾ By using polyethylene glycols as carriers it is possible to give simultaneously both water-soluble added substances (e.g., colchicine, *Setälä*) and the ordinary fat-soluble carcinogen.

The mice gained in weight during the experiment suggesting that the medium is well tolerated. It also appeared in another connection that e.g., »Carbowax 1500« in aqueous solutions is non-toxic to *Parameciae* (unpublished data). When the experiments terminated in the present series no symptoms were seen indicating lymphatic proliferations as sometimes has been mentioned in the literature.^{8, 9, 18, 20, 21)}

The investigations are being continued.

Grateful acknowledgment is made to Prof. R. Collander, Ph. D. for valuable advice during the course of this work, to Miss Kerttu Elg, assistant apothecary, for the preparation of the compounds used, and to Mrs. Ida Turunen for the care of the experimental animals.

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Sept. 4, 1948.

50 applications (3 x wly) of MC (0.36%)
in acetone.



Fig. 1.

Mouse 38/1 treated with 20-methylcholanthrene dissolved in acetone.
Macroscopically malignant tumour.

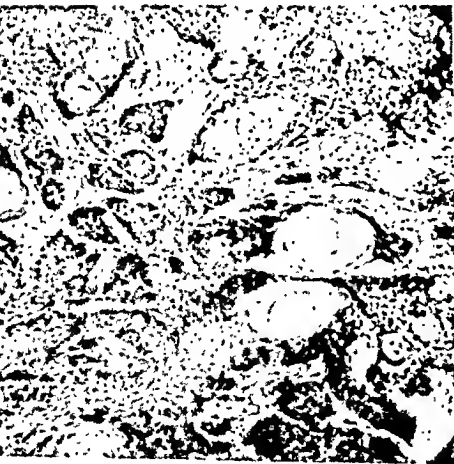


Fig. 2.

Biopsy specimen from the tumour
seen in Fig. 1. Squamous cell carcinoma with cornification.

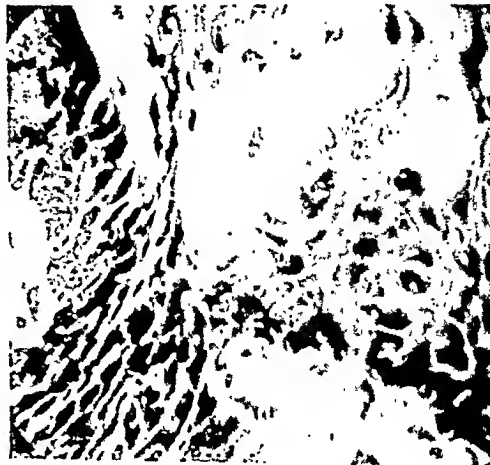


Fig. 3.

Same section as in Fig. 2. Higher
magn.

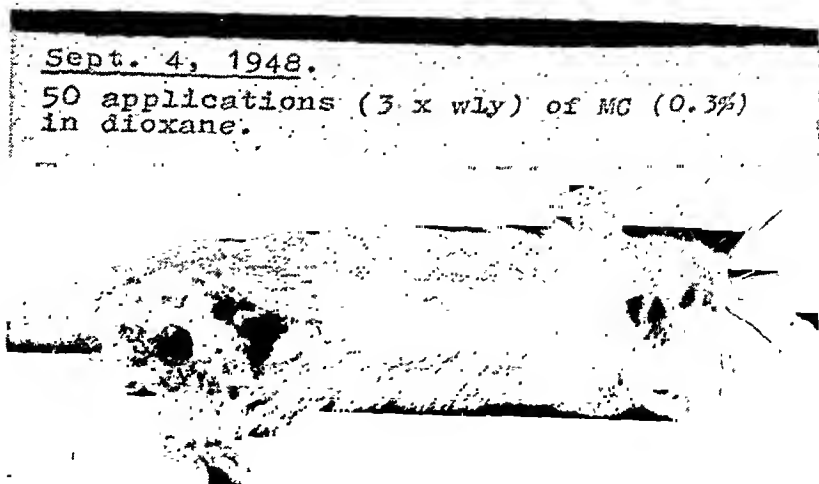


Fig. 4.

Mouse 33/I treated with 20-methylcholanthrene dissolved in dioxane.
Macroscopically not certainly malignant.



Fig. 5.

Biopsy specimen from the tumours seen in Fig. 4. — Squamous cell carcinoma with cornification and parakeratotic changes.



Fig. 6.

Same section as in Fig. 5. Higher magn.

July 28, 1948.

44 applications of DMBA (0.25%)
in "Carbowax-1500". 3 x wly.

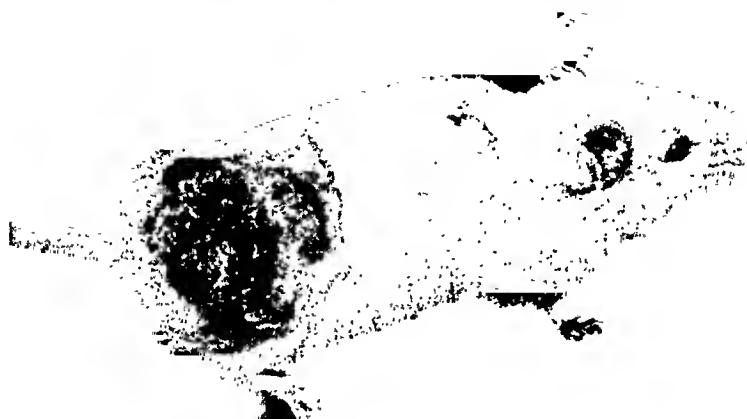


Fig. 7.

Mouse 35/X treated with 9,10-dimethyl-1,2-benzanthracene in »Carbowax 1500«. Macroscopically malignant.

Sept. 4, 1948.

60 applications (3 x wly) of DMBA (0.25%)
in "Carbowax-1500".



Fig. 8.

Mouse 35/I treated with 9,10-dimethyl-1,2-benzanthracene dissolved in «Carbowax 1500». Macroscopically not certainly malignant tumours.



Fig. 9.

Biopsy specimen from the tumours seen in Fig. 8. — The tumour growth reaching to the panniculus adiposus and to the muscle layer.



Fig. 10.

Same section as in Fig. 9. Squamous cell carcinoma with cornification.

Sept. 4, 1948.

129 applications (6 x wly) of MC (1.0%)
in "Carbowax-1500".



Fig. 11.

Mouse 24/1. Cutaneous tumour with macroscopically malignant properties.
Treatment with 20-methylcholanthrene in »Carbowax 1500«.



Fig. 12.

Biopsy specimen from the tumour
seen in Fig. 11. Squamous cell carcinoma
with parakeratotic changes.

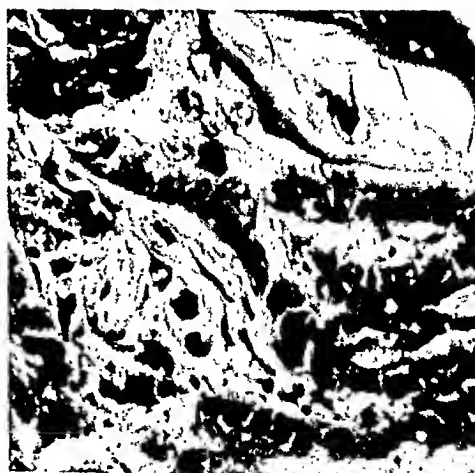


Fig. 13.

Same section as in Fig. 12. Higher
magn. Numerous mitotic figures.

Sept. 4, 1948.

Subcutaneous injection (May 9, 1948)
of 2.0 mgm of DMBA
in 0.5 ml aqueous "Carbowax-1500".



Fig. 14.

Mouse 10/I. Tumour consisting of both carcinomatous and sarcomatous components occurring after subcutaneous injection of 9,10-dimethyl-1,2-benzanthracene in aqueous »Carbowax«.



Fig. 15.

Biopsy specimen from the tumour seen in Fig. 14. Squamous cell carcinoma with cornification.

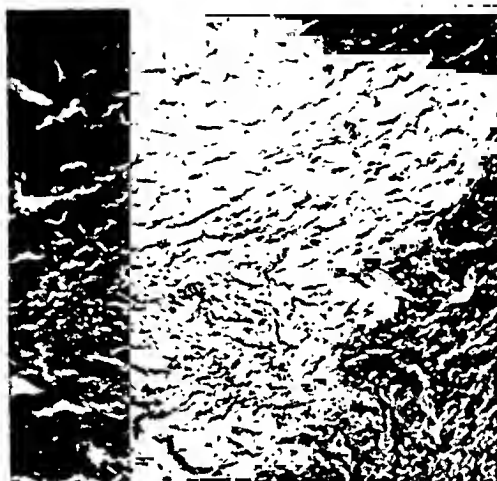


Fig. 16.

Spindle cell sarcoma. Biopsy specimen from mouse 20/I. The tumour occurring after subcutaneous injection of 20-methylcholanthrene in aqueous »Carbowax«.

EXPERIMENTAL IMMERSION-FOOT IN RABBITS

By *Leiv Kreyberg*.

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In a previous paper the author (1946) stated that, in spite of the known fact that long exposure to moderate cold is the background of immersion-foot, trench-foot and similar conditions the development of these conditions has not been observed from hour to hour during exposure, so that our conception of their development is based upon fragmentary information, and our conclusions are drawn from analogy.

Th. Lewis (1942) in an experimental study on man concluded that, when an extremity is cooled, as by immersion in cold water (5°C) it swells. This increase of volume occurs both in the skin and subcutaneous tissue, and may within 3 hours amount to as much as 15 % of the original volume. The swelling is due mainly to an edema of the tissues, judged to be inflammatory from its relatively rapid outpouring and from its relatively high protein content. The contribution in the form of imbibed water is very slight. From this and correlated evidence it seems that cold directly injures the skin and subcutaneous tissues. This effect begins at about 15° to 18°C . and increases as the scale of temperature is descended.

Brown, Wise and Wheeler (1947) confirmed these observations, using a more refined technique, the pressure plethysmograph. They also confirmed an older observation by *Landis and Gibbon* (1933), that cold reduced the rate of reabsorption of previously filtered fluid. They found that this disturbance of fluid removal is compatible with a reduction of the effective colloid osmotic pressure of the plasma amounting to approximately 10 cm water.

Brown and Landis (1947) on the other hand concluded that during local cooling of the frogs mesentery, no signs of capillary injury or of leakage of protein from the capillaries appeared until after the tissue had been frozen.

Lange, Weiner and Boyd (1948) exposed the hind legs of rabbits

to ice-water and found, that after immersion, the temperature goes down rapidly, but does not reach the temperature of the surrounding water even after several days. After intravenous injection of fluorescein a delayed and less concentrated staining of the cooled area is found, as compared to the normal side. Severe swelling occurs during the exposure, it is quite noticeable after 24 hours, and severe after forty-eight hours.

The occurrence of edema after prolonged moderate cooling has been an «everyday» observation in populations living in cold climates.

The information about the early vascular responses to local cold is, however, still fragmentary. The brief summary of the experiments reported upon above shows that evidently the response of different animal species varies, if not in principle, at least in degree.

The present experiments in many respects follow the technique described by Lange, Weiner and Boyd, but a few more details have been investigated by a combination of different experimental methods.

EXPERIMENTS

Technique.

Rabbits have been used as experimental animals. The animal material has deliberately been made non-uniform with the purpose of seeing the relevant reactions as far removed from individual variations of response, as possible. In the experiments were used animals of both sexes, of a series of different coat colours and of varying age and weight. All animals were, however, in a very good nutritional state. As a matter of economy the same animal has been used for different observations during one experiment, and in several animals both legs were used at different periods.

Exposure to cold. The animals were strapped to a structure, as shown in Fig. 1, with the hind limbs projecting through holes in the bottom. One leg at the time was immersed in a tall square glass jar filled with water and ice cubes. If plenty of ice is present the temperature in the water ranges between 2° C. and 5° C. If the ice is nearly melted and only fragments are present, the temperature may come nearly up to 8° C. and probably is still higher very close to the limb. Great care has therefore been exerted to see that the glass contains plenty of ice, and that the cubes always are close to the skin of the limb. A constant personal vigilance has been enforced, even in the prolonged experiments. (A technical apparatus, as described in the experiments of Lange, Weiner and Boyd is out of the question in our laboratory, owing to lack of funds and technical facilities). Any interruption of the continuity of the exposure will lead to confusing results. The limb was immersed in the ice-water up to, and including the lower third of the thigh. The hairs of the whole leg and thigh were removed, usually by shaving, both on the experimental and on the control side. This was done in order to observe the vascular changes, as well as to obtain a stronger exposure.

The skin temperature was measured by a thermocouple (la Pyrometric Industrielle, Paris) with a contact disc of 15 mm diameter. The reading was quick and direct in centigrades.

Edema was diagnosed in different ways, partly through direct observation of the swelling of the limb and the thickening of the skin, partly by measuring the circumference of identical niveaus of the exposed and the control limb. Finally the edema was diagnosed at autopsy and confirmed

by microscopy. After 24 hours exposure, the edematous fluid may be collected through puncture.

Intravenous injection of lithium-carmin (2.5 %) was used as one of the means of obtaining information about the vascular reactions and the state of the blood circulation in the exposed area (Kreyberg and Rotnes, 1931). An increased staining of the tissue by this colloidal dye may be caused either by an increased blood flow, even with the maintenance of normal permeability, or by an increased permeability in spite of normal, or even reduced blood flow. A combination of increased permeability and an increased blood flow

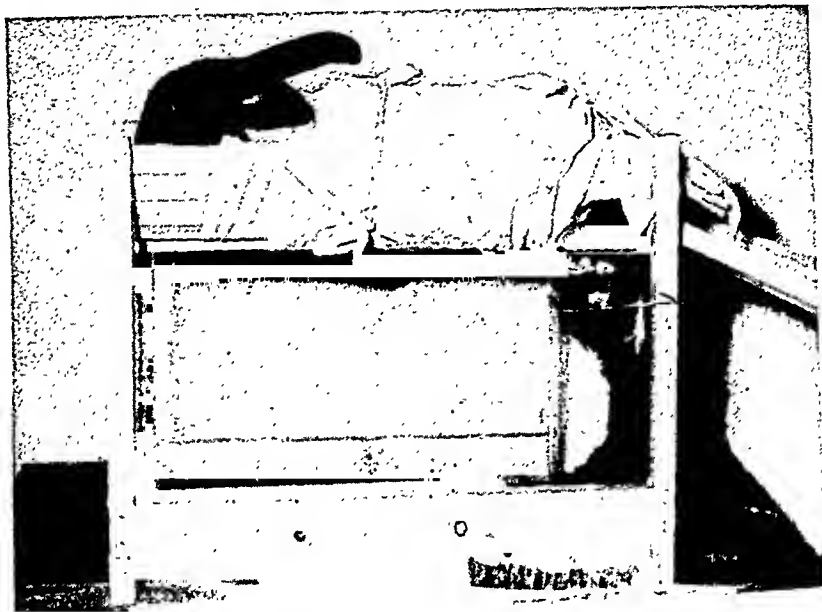


Fig. 1.

will evidently give the strongest response. The observation of an increased staining will therefore have to be supplemented by other methods to give a full answer.

Intravenous injection of fluorescein («Fluorescite», C. F. Kirk, Co, N. Y.) as described by Lange and Boyd (1943) was used in combination with the other methods. This dye has a much smaller molecule and enters the tissue quicker and in larger amounts than lithium-carmin. This ingenious method also gives a quick reply as to the admittance of the streaming blood into a tissue area. Any mechanical or functional hindrance to the blood will prevent the dye from entering the area, and this area will stand out sharply against the brilliant yellow surroundings. The glass of the jar holding the ice-water in this experiment had a composition, which permitted practically all the ultraviolet rays, used to show the fluorescence, to penetrate. Numerous observations showed, that it made no material difference for the diagnosis, whether the limb was lifted out of the jar, or remained in the ice-water. This is of great importance, as the observation can be carried out continuously, without any disturbing interruptions of the exposure.

Subcutaneous injections of fluorescein (0.5 cc) was used to obtain information about the return of the tissue fluid into the general circulation. The local deposit can easily be seen through the skin, under ultraviolet light.

The diffuse spread, as well as the filling of the lymphatic vessels can be followed in detail, and when the dye is resorbed in sufficient quantities, it can be diagnosed in the general circulation by the staining of the skin, mucosas, and especially the eyelids. Tests were performed with drops of blood from the ear, in the hope of getting an easy test for the amount in the general circulation, but it turned out, that the observation of the eyelids was a finer and more reliable method.

Autopsies under ultraviolet illumination were performed in some of the animals after local injection of fluorescein. The local deposits, as well as the spread through the blood vessels and the lymphatic channels can be followed in detail. The excretion through the kidneys and the liver can also be followed step by step, including the passage from the gall bladder into the small intestines, through peristaltic movements.

Observations during the period of exposure.

Within a few seconds after the immersion of the leg into the ice-water, some animals show a local *tonical spasm* of the skeletal and the skin muscles. A similar reflex can also be observed on the control side.

Table I.
(Edema formation.)

Rabbit no.	Length of exposure	Edema
KK 1/48 ♂, albino 1 800 g	4¾ h (1015—1500)	No edema (right leg)
KK 11/48 ♀, black 3 010 g	5 h (0945—1445)	Edema (left leg)
KK 5/48 ♀, albino 4 500 g	5¾ h (0900—1445)	Considerable edema (left leg)
KK 10/48 ♂, »wild type« 2 850 g	6 h (0900—1500)	Moderate edema (left leg)
KK 2/48 ♂, albino 3 200 g	6 h (0920—1520) 8 h (0920—1720)	Edema Marked edema
KK 3/48 ♂, silver 4 200 g	12¼ h (0900—2115)	Marked edema Circumference: exposed side 11 cm (left) (thigh) control side 9,5 cm (right)
KK 17/48 ♀, albino 2 800 g	23½ h (0830—0800)	Very strong edema Circumference: exposed side 14,5 cm (right) (thigh) control side 12 cm (left)

In most animals, but to a varying degree, *fibrillary contractions* of the skin and the skeletal muscles can be seen after half an hours exposure, and the contractions may continue for as long a period as any experiment in this series has been carried.

The colour of the skin during the first 15 to 30 minutes of immersion is slightly cyanotic, but later it turns bright pink, and stays so for the rest of the experiment, with the occasional interruptions of white spots, which may come and go from time to time. After 18 to 24 hours exposure the edema gives the skin a pasty whitish-pink appearance.

Edema appears at slightly different times in the various animals, but after 5 hours exposure all animals examined have shown a definite edema, as indicated in Table I.

Histological preparations show that the collagene bundles of the corium are separated by fluid, and a gelatinous tissue is found between the skin and the superficial fascia. Also the skeletal muscles show edema.

The arterial blood flow in the exposed tissue has been examined by two different techniques, viz. the measuring of the skin temperature and the observation of the penetration of fluorescite into the tissue.

a) The skin temperature to a certain degree indicates the rate of the arterial blood flow. In a series of animals the skin temperature was measured. The limb was lifted out of the ice-water, dried quickly and very gently with fine cellulose, avoiding delay and massage. The temperature was read within 10 to 15 seconds after the limb was out of the water. The results of the readings are collected in Table II.

Table II.
(Skin temperature).

Rabbit no	Length of exposure	Temperature		
		Exposed leg	Control leg	Room
KK 16/48 ♀, albino 4 500 g	1 h	10,5° C	32° C	22° C
	2 h	10°	32°	22°
	12 h	9,5°	30°	21°
KK 17/48 ♀, albino 2 800 g	1 h	9,5°	31,5°	22°
	2 h	9°	31°	22°
	12 h	9,5°	30°	21°
	24 h	12°	27°	18°
KK 14/48 ♀, albino 3 700 g	7 h	11°	30°	21°
	11 h	11°		22°
KK 11/48 ♀, black 3 010 g	8 h	11°	32,5°	23°

Results: In the course of one hour the superficial skin temperature is very close to 10° C, and stays so, remarkably constant, up to 24 hours, the longest period examined. This is in complete agreement with the findings of Lewis, and Lange, Weiner and Boyd. The temperature of the other leg drops 2 to 4 degrees centigrade from the normal, and usually stays so for the period examined.

b) *Penetration of fluorescein into the superficial vessels of the skin.* After intravenous injection of fluorescein the mucosas and the normal skin turns yellow in a couple of seconds. The entrance of the stain into the cooled limb was observed after varying time of exposure. The observations are collected in Table III.

Table III.
(Intravenous injection of fluorescein).

Rabbit no	Length of exposure when injected	General staining (mucosa and skin)	Development of the staining of the exposed limb
KK 15/48 ♀, albino 4 590 g	1 h (1000—1100) (1230 h a new injection of fluorescein).	Immediately	1115 h small yellow spots 1200 h no change 1300 h no change 1500 h still small yellow spots only
		Immediately	1105 h small yellow spots 1130 h spots slightly larger 1200 h slight enlargement of the spots 1230 h spots a little larger and some confluence. $\frac{3}{4}$ of the area unstained 1400 h no change 1430 h nearly $\frac{1}{3}$ of the area yellow 1530 h no change
KK 18/48 ♀, albino 4 900 g	2 $\frac{1}{4}$ h (0845—1100) (right leg)	1300 h good staining 1430, same	
		Immediately	1510 h a few yellow spots in the upper parat 1530 h possibly a few more spots and deeper staining 1600 h some increase of staining, but still large areas unstained 1630 h some increase and confluence of spots 1700 h no change
KK 13/48 ♀, silver 3 090 g	6 h (0900—1500)		
		Immediately	
KK 3/48 ♂, silver 4 200 g	9 $\frac{1}{4}$ h (0900—1815)	Immediately	1320 h small spots stained 1845 h spots are larger and staining deeper
		2015 h still good staining	1915 h still some spots unstained 2015 h still small spots unstained

Rabbit no	Length of exposure when injected	General staining (mucosa and skin)	Development of the staining of the exposed limb
KK 19/48 ♂, albino 3 050 g	12 h (0830—2030)	Immediately	2035 h a little yellow on the back of the thigh 2040 h a faint diffuse yellow colouring, not in small spots 2050 h increasing intensity 2110 h a distinct yellow marbeling on the leg 2130 h nearly 75 % of the area bright yellow with darker spots very like the control side
KK 18/48 ♀, albino 4 900 g	15 h (0830—2330) (left leg)	Immediately	2332 h large yellow spots 2335 h approximately 1/4 of the area yellow 2345 h increasing colouring 1/3 of the area 2400 h a dense and small spotted colouring of half of the area 2430 h increasing staining, but still unstained areas
KK 17/48 ♀, albino 2 800 g	18 h (0830—0230)	Immediately	0231 h large yellow areas 0235 h larger areas yellow than not yellow 0240 h all stained except small area outside the knee

Results: During the first hours of exposure a spasm is established in the terminal arterioles and venules of the skin to the degree of nearly completely excluding any entrance of fresh blood to the superficial capillary network. The second to fourth hours of exposure show a very strong maintenance of this condition, but gradually from the sixth to the twelfth hour of exposure this hindrance is lifted and an animal exposed for 18 hours shows a complete staining, i. e. entrance of fresh blood in the course of 10 minutes after intravenous injection. This is, however, still a reduced inflow of fresh blood, as compared to the normal limb.

Removal of tissue fluid is normally effected through a combined process of resorption through the smaller veins and through a lymphatic drainage. This process of removal has been examined by the *injection of fluorescein subcutaneously*. Through a long and fine canula, 0.5 cc of the dye was injected into the loose connective tissue between the skin and the superficial fascia. A series of control observations were made in the normal leg of the same experimental animal, or in other rabbits. The results of two control experiments are summarized in Table IV a.

Table IV a Control series.
(Subcutaneous injection of fluorescein).

Rabbit no	Injection	Local development	Appearance in general circulation
KK 12/48 ♀, albino 4 800 g	1010 h right leg	1010 h a complete yellow disc, oval 2×3 cm diameter	Eyelid faint yellow?
		1011 h fine lymphatic extensions	
		1014 h centre of disc dark and a 1 mm wide yellow border	
		1020 h upper part of disc shows a ragged contour	
		1025 h a 1 cm wide yellow border and long lymphatic extensions	
		1040 h very long lymphatic extensions towards groin and anal region	
		1055 h	
KK 14/48 ♀, albino 3 700 g	1905 h (left leg)	1110 h dark centre, 1,5 cm in diameter, and a great diffuse spread of the stain	Eyelids definitely yellow Marked staining of eyelids
		1905 h a complete yellow disc	
		1910 h lymphatic spread	
		1915 h upper part shows ragged contours	
		1920 h a diffuse and considerable spread	
		1935 h yellow area greatly increased and large lymphatic vessels	
		1950	
			Eyelids faint yellow?
			Eyelids and lower part of the leg yellow

Autopsies under ultraviolet illumination were likewise performed in a series of control animals in order to follow the rate and the roads of resorption of the stained fluid. The results are collected in Table IV b.

Table IV b Control series.

Rabbit no	Duration of resorption	Observations at autopsy
KK 25/48 ♂, grey 2 100 g	7 minutes (1016—1023)	Considerable local spread with marked staining of the lymph vessels extending to the thoracic duct. Liver and gall bladder yellow, kidneys intensely stained, urine stained. Local blood vessels well stained.
KK 9/48 ♂, silver 2 850 g (left leg)	1 hour (1025—1125)	General staining of the skin and mucosas. The lymph vessels including the thoracic duct intensely stained. Kidneys, urethters and bladder intense yellow. Liver gall bladder and 20 cm of the small intestine yellow. The local blood vessels show a very faint staining.

Results: The observations in vivo and by autopsy showed, that during the injection a few lymphatic vessels may be filled mechanically under the pressure of the injection, but the main bulk of the dye is deposited as a strictly localized mass, which immediately forms a brilliant yellow disc under ultraviolet illumination. During the next minutes, the centre of the disc often turns dark, and a fine border,

Table IV c. *Exposed series.* (Ctr. IV a).

Rabbit no	Length of exposure when injected	Injection	Local development	Appearance of dye in general circulation
KK 20/48 ♀, albino 4 600 g	1 h (1000—1100) (left leg)	1100 h	1105 h sharp limitation, a few lymphatics filled 1115 h more lymphatics 1200 h a well developed network of lymphatics, Diffuse limitations 1300 h increasing ramification and more ragged outlines 1400 h no change 1530 h a slow peripheral spread	No staining No staining No staining No staining (4½ h)
KK 5/48 ♀, albino 4 500 g	3 h (0900—1200) (right leg)	1200 h		1445 h no general staining (2¾ h)
KK 11/48 ♀, black 3 010 g	5 h (0945—1445)	1445 h		1645 h no general staining (2 h)
KK 20/48 ♀, albino 4 600 g	10 h (0900—1900) (right leg)	1900 h	1910 h disc expanding slowly, fine lymphatics 1920 h ragged outlines all round 1930 h many distinct vessels 2000 h very ragged outline, especially towards the groin 2030 h increased staining 2045 h very widespread staining 2100 h no change	No staining No staining Faint staining of eyelids? No staining (2 h)
KK 16/48 ♀, albino 4 500 g	20 h (0830—0430)	0430 h	0434 h upper contours ragged 0445 h disc is spreading, several lymphatics visible 0530 h further peripheral spread 0800 h strong local diffusion	No staining No staining (3½ h)

Table IV d, *Exposed series.*
(Cfr. IV b).

Rabbit no	Duration of exposure	Duration of resorption	Observation at autopsy
KK 11/48 ♀, black 3 050 g (right leg)	1 hour (1050— 1150)	7 minutes (1150— 1157)	Considerable local diffusion. Local lymph vessels stand out brilliantly stained, including the iliac lymph nodes. From the branching off of the kidney vessels no staining. Kidneys, bladder and gall bladder not stained. Thoracic duct not stained. Local blood vessels not stained.
KK 26/48 ♀, albino 2 900 g	2¼ hour (0930— 1145)	1 hour (1045— 1145)	Considerable local spread. Larger lymphatic vessels intense yellow, including the thoracic duct. The kidney surface shows a stellate yellow staining. Urine yellow. Liver and gall bladder faint yellow. Intestines not stained. Local veins faint yellow.
KK 27/48 ♂, »wild t.« 3 220 g	9 hours (0900— 1800)	7 minutes (1753— 1800)	A definite edema in the cooled area. The local spread is moderate. Lymphatic vessels are stained up to the liver, including swollen iliac nodules. Kidneys and gall bladder not stained. Thoracic duct not stained.
KK 28/48 ♀, alb. 3 950 g	24 hours (0815— 0830) (next day)	7 minutes (0823— 0830)	A rather quick local spread of the dye, like »masses of smoke« under the skin (edema). No lymphatics visible. At autopsy no lymph vessels stained, not even the iliac. No staining of the kidneys, urine, liver or gall bladder.

a few millimeter wide only, remains yellow. In the course of the following 5 to 10 minutes, the contours of the disc begin to be ragged, and the dye is diffused into the surrounding connective tissue. At this moment the larger lymphatics can be seen as clear yellow lines under the thin epidermis.

At autopsy the regional lymphatic drainage can easily be followed. In a normal rabbit under standard control conditions, the lymphatic vessels alongside aorta become intensely stained all the way up to, and including, the thoracic duct, in the course of 7 minutes, or less. After 7 minutes the dye has reached the kidneys in a sufficient concentration to give the urine in the bladder a distinct fluorescence. Likewise the gall-bladder is stained. After 45 to 60 minutes the dye has passed into the general circulation in quantities sufficient to stain the eyelids and the mucosas, and the excretion of the stain can be seen a good way down the small intestines, following the peristaltic evacuation of the intestinal contents.

Similar experiments were performed on the *exposed limb*, the sub-

cutaneous injection being given after different periods of exposure. The results are summarized in Tables IV c and IV d.

Results: The local spread and the resorption in the cooled limb is greatly retarded. In the animals with a long exposure and heavy edema, the character of the local spread is to a certain degree influenced by the edematous fluid. The filling of the lymphatic vessels seems to be hindered, and the stain more stagnant in the edematous tissue. This condition seems to be constant, or increasing, in contrast to the opposite process, that of entrance of fluid into the local blood vessels, which is more abundant after 12 to 24 hours than at the beginning of the cooling.

Vital staining with lithium-carmin. In a series of animals staining of the skin by lithium-carmin after intravenous injection has been observed. The results are summarized in Table V.

Table V.
Vital staining with lithium carmin during exposure.

Rabbit no	Total exposure	Length of exposure before injection	Period of staining	Local staining of the tissue	Edema
KK 10/48 ♀, *wild t. 2 800 g	6 h (0900-1500)	3 h (0900-1200)	3 h (1200-1500)	0	Moderate
KK 6/48 ♀, albino 3 000 g	12 h (0930-2130) (right leg)	0 h	12 h (0930-2130)	0	Considerable
KK 13/48 ♀, silver 3 090 g	8 h (0900-1700)	4 h (0900-1300)	4 h (1300-1700)	Faint ?	Some
KK 14/48 ♀, albino 3 700 g	12 h (1000-2200)	4 1/2 h (1000-1430)	7 1/2 h (1430-2200)	Faint	Considerable
KK 3/48 ♂, silver 4 200 g	12 1/4 h (0900-2115) (left leg)	9 h (0900-1800)	3 1/4 h (1800-2115)	0	Circumference left: 11 cm right: 9.5 cm
KK 17/48 ♀, albino 2 800 g	23 1/2 h (0830-0800) (right leg)	18 h (0830-0230)	5 1/2 h (0230-0800)	Strong	Circumference right: 14.5 cm left: 12 cm

Results: During the first 12 hours a very low concentration of lithium-carmin is found in the tissue of the cooled limb, even after the occurrence of edema. To obtain a staining of the tissue stronger than that on the control side a prolonged cooling is necessary.

From the 12th to the 24th hour of exposure, however, an increasing

staining can be seen and after 18 to 24 hours of exposure, the staining is much stronger than on the control side.

The specific gravity of the cell-free edema punctate was examined after Phillips and van Slyke (1945) in a single case (rabbit KK 16/48) after 22½ hours exposure. The specific gravity was 1.011, corresponding to a protein content of 1.44 g pr. 100 cc.

Discussion and Conclusions.

The experiments reported upon give a rather clear picture of the character of the early circulatory changes in the skin of rabbits during the local exposure to moderate cold.

Nearly immediately after the immersion into the ice-water, a local spasm of the terminal arterioles and venules of the skin occurs. At the same time a dilatation of the superficial minute vessels takes place, whereby a stagnant mass of blood is captured in the surface vessels. After a couple of hours the spasm is slightly reduced, permitting a trickle of fresh blood to enter the superficial vessels. The spasm continues to be less complete, but even after 18 to 24 hours the circulation is greatly reduced as compared with the normal side.

Corresponding to the reduced inflow of fresh blood to the superficial vessels, the fluid resorption and lymphatic drainage is greatly reduced. But whereas the entrance of fluid increases during the cooling and is remarkably augmented after 24 hours of exposure, the drainage of fluid is still very poor, and possibly even decreased.

The constant temperature of the skin points to a regular blood flow in the deeper layers, probably effected through direct arterio-venous connections.

Edema occurs after some 4 to 6 hours cooling, and this edema is probably mainly the result of the reduced fluid resorption and drainage. The main support for this view is the very poor circulation on one hand and the complete absence of staining by lithium-carminine, on the other hand. After 12 hours, or more, the edema changes in character, lithium-carminine appearing in the fluid. After 18 to 24 hours exposure the staining by lithium-carminine is very marked. This strong staining cannot be explained by the increasing influx of fresh blood in the cooled area, only, as the staining is much stronger than on the control side, in spite of the better circulation on that side. The increased staining is caused by an increased vascular permeability. The high protein content of the edematous fluid confirms this conclusion.

The mechanism of the vascular responses during the first 24 hours of cooling in the rabbit seems to be a twofold: First, a functional, physiological response in the form of an adaptation through vasoconstriction. This results in a moderate edema, probably mainly caused by a hindered resorption and drainage from the constricted vessels. Second, a pathological response to tissue damage, with increased vascular

permeability, and a much stronger edema. This second response represents the early vascular phase of inflammation. The full inflammatory reaction is, however, through continued cooling, checked by the vascular spasm.

The observations recorded here, and the explanation advanced in this discussion hold good for the rabbit. The observations, as well as the explanation, deviates from the conditions for man, as put forward by Lewis, and Brown, Wise and Wheeler. In man the edema seems to be rich in protein from the very beginning of the cooling. In the frog, on the other hand, Brown and Landis do not find any increased permeability. The rabbit, therefore seems to be in an intermediate position. This animal is adapted to wet and damp ground, and without any artificial protection. We may subject the rabbit to a cooling, which would give man, the tropical animal, severe tissue damage, but the rabbit will show moderate changes. A few hours in ice-water will produce a marked inflammatory edema in man, no reaction in the frog, and a light physiological response in the rabbit. The protracted reaction in the more cold-resistant rabbit, may reveal a series of stages, which may be present also in man, but difficult to observe, because of the rapid sequence.

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SENSITIVITY OF HEMOPHILUS DUCREYI TO PENICILLIN, STREPTOMYCIN AND SULFATHIAZOLE

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In the course of systematic investigation into the bacteriology of *Hemophilus Ducreyi*, a determination was made of the sensitivity of this microbe to penicillin, streptomycin, and sulfathiazole. Very few examinations have hitherto been published about this subject, and as the greater part of these examinations were performed with old laboratory strains it has been found expedient to present the results of a determination of the sensitivity for strains dried immediately after their isolation ad modum Flosdorff & Mudd.

The following previous works on the in vitro sensitivity have been available:

Mortara, F. & R. R. Feiner & E. Levenkron in 1944 examined the penicillin sensitivity of 5 strains and found the same sensitivity as for *staphylococcus aureus* (test strain No. 209). 2 strains were old laboratory strains, 3 were freshly isolated. Cultivation was made at 35° C. partly in fluid medium, 10 % rabbit serum bouillon, and partly in solid medium consisting of agar with 10 % defibrinated rabbit blood. Readings of the tests were taken after 24—48 hours. The *Ducrey* bacillus grew on these media at 0.03 units/ml concentration, but not at 0.13 units/ml.

Tung, T. & C. N. Frazier (1945) found for four laboratory strains that they were slightly less sensitive than *staphylococcus aureus* (test strain No. 209). The medium consisted of »Difco brain-heart infusion broth« + 10 % defibrinated rabbit blood. Incubation was made at 34° C, and reading after 48 hours. The range of growth was + growth at 0.10 units/ml, but 0 growth at 0.15 units/ml.

Vilanova, X. and G. Rey (1945) in a very short article stated that the penicillin resistance of the *Ducrey* bacillus was so high that penicillin could be used in selective media. However, only one cultivation experiment was made, and the exact penicillin concentrations were not stated.

Mortara, F. & M. T. Saito (1946) also published a more extensive examination of the sensitivity of 7 strains. Five strains were isolated during two years before the examination and kept by weekly reinoculations in defibrinated rabbit blood. The other two strains were laboratory strains. In this

work the media were partly fluid: »Bacto nutrient broth« + 20 % horse serum + 0.5 % NaCl, and partly solid »Bacto nutrient agar« + 20 % citrate horse blood + 0.5 % NaCl. The solid medium was used in Petri dishes which were closed airtight with »parafilm« in order to avoid desiccation. Incubation was made at 35° C and reading after 24—48 hours' incubation. The results, both for streptomycin and sulfathiazole, were that the Ducrey bacillus showed slightly higher sensitivity than hemolytic streptococci.

The following works elucidate the in vivo sensitivity of the Ducrey bacillus to the different chemotherapeutics:

Pereyra, J. A. & S. Landy (in 1944) examined the effect of penicillin on experimentally produced chancroid in 3 volunteers, and no effect whatever was found. It should be noted, however, that infection was made with large quantities of bacilli and the penicillin doses administered were rather small. Thus 1 patient was given 80,000 units per 24 hours (totalling 600,000 units), and the other two got only 40,000 units per 24 hours (total 560,000 units). *L. A. Day* (1945) found excellent effect in a case which was cured by a total of 100,000 units (20,000 \times 5 intramuscular at 3 hours' interval). *Vilanova et al.* likewise found good effect, as their patient was cured by penicillin applied locally in the form of ointment (dose not stated).

No works have been published on streptomycin treatment of patients, but *Mortara et al.* (1947) found excellent effects of this antibiotic in the treatment of chancroids induced experimentally in rabbits.

On the sulfathiazole sensitivity the following is available:

A. Kristjansen (1939), *O. S. Culp* (1940), *B. A. Kornblith & A. Jacoby & L. Chargin* (1941), *V. C. Harp* (1946), *D. G. Lao & M. D. Trussell* (1947), and others found excellent effect of sulfathiazole in their patients, whereas for instance *T. B. Turner & T. H. Sternberg* (1944) only found effects in about 50 % of their cases. The last mentioned authors point out, however, that the diagnosis was not certain because it had been made without any aids whatever.

Author's investigations.

The aims have been, partly to determine the in vitro sensitivity by cultivation on agar plates and partly to obtain an impression of the relation between the found in vitro values and the in vivo sensitivity by penicillin treatment of chancroid induced in two volunteers.

In vitro sensitivity.

Strains. A total of 14 strains have been investigated, 12 of which were isolated by cultivation from chancre pus, and 2 (A_1 and A_2) from bubonic pus. After isolation, the strains were dried ad modum Flossdorff & Mudd and kept in vacuum ampoules at + 4° C. At the time of the examination, the oldest strains were 18 months old, whereas the youngest were 6 months old (11 strains). In regard to the procedure adopted for isolation and drying I refer to my previous publication on this subject.

For test strains were used *staphylococcus aureus* (Oxford test strain No. 209) and *streptococcus hemolyticus*, group A, type 1. These strains have been cultivated under exactly the same conditions as the Ducrey strains. In one preliminary experiment it was found that the *staphylococcus* strain showed the same sensitivity to penicillin as two freshly isolated strains from cases of furunculosis.

Media. For preliminary experiments both fluid and solid media were used.

Two different fluid media were tried. The first one consisted of bouillon with 20 % defibrinated sheep blood. This medium was inoculated after sedimentation of the erythrocytes, and the growth took place on the surface of the layer of erythrocytes. The other medium consisted of semifluid agar with 30 % defibrinated sheep blood. This medium was of such a consistency that a clear layer of about $\frac{1}{2}$ centimetre was formed at the top of the medium when sedimentation had taken place. The growth took place at the level where this layer met the blood.

For solid medium was used 30 % defibrinated sheep blood in agar plates (nutrient agar with 1 % peptone) in Petri dishes. As saturated water vapours are required to obtain growth on this medium the dishes were placed in closed glass jars with a surplus of water on the bottom.

It soon appeared, however, that the fluid media were unsuitable for the purpose because the results varied from one experiment to the other; nor could any reliable impression be gathered of the differences in the sensitivity of the strains. The limits for the range of growth in fluid media stated by *Mortara et al.* (1946) also show a very wide margin; as regards streptomycin sensitivity, for instance, 10 units of streptomycin per ml medium will inhibit growth altogether, whereas growth was found in 95, 58 and 17 % respectively of the tubes containing 5, 1, and 0.5 units per ml.

Another reason why cultivation on solid medium was thus preferred was that the growth is much better than in fluid media; moreover, the results of cultivations can be read macroscopically. Finally, the variations in the results of each experiment are quite insignificant.

Chemotherapeutics: The chemotherapeutics were:

Penicillin, used in the form of the Na-salt of G-penicillin made by the »Leo« factory, Denmark.

Streptomycin, made by Merck & Co., U. S. A., and

Sulfathiazole in the form of »chemosept« (2 (p-aminobenzol-sulfonamido) thiazole) from the »Ferrosan« factory, Denmark.

The dissolved substances were added to the media in the doses stated in the tables.

Inoculation. The plates with the different chemotherapeutics were

inoculated on the same day as they were made. The preliminary culture was cultivated on plates inoculated with dried culture from an ampoule.

Varying amounts of inoculum were used in preliminary experiments on penicillin sensitivity, and it was found that the amount was of great importance. At a certain thin inoculation, growth occurred only on plates containing 0.02 or 0.04 units of penicillin per ml of medium, and the figures for sensitivity were seen to decrease with increasing inoculum to a certain limit. In order to obviate these potential errors it was then preferred to give an inoculation which was just sufficient to give confluent growth on the control plates.

The plates were inoculated with a drop from a tube of bouillon in which 2—4 colonies from the preliminary culture had been suspended. Before suspension the colonies were crushed between two sterile slides.

Results. All the results stated in the tables were found in experiments with cultivation on plates, which were incubated for 3 days at 33—34° C. The conditions of cultivation have been found optimal in previous experiments of which a summary was published in 1947.

Explanation of tables: 0 means no growth; + means growth ranging from confluent growth on the control plates to individual colonies on the plates with the highest concentrations.

The sensitivity of the test strains is treated in the discussion.

Table I.
In vitro sensitivity to penicillin.

Concentration Strain	0.32 U./ml.	0.16	0.08	0.04	0.02	Control
A1	0	+	+	+	+	+
A2	0	+	+	+	+	+
129	0	+	+	+	+	+
133	0	+	+	+	+	+
134	0	+	+	+	+	+
140	0	+	+	+	+	+
146	0	+	+	+	+	+
155	0	0	0	+	+	+
156	0	+	+	+	+	+
161	0	+	+	+	+	+
167	0	+	+	+	+	+
I	0	+	+	+	+	+
IV	0	0	0	+	+	+
V	0	0	0	+	+	+
Staph. aureus	+	+	+	+	+	+
Hem. Strep.	0	0	+	+	+	+

Table II.
In vitro sensitivity to streptomycin.

Concentration Strain	5 U./ml.	2.5	1.25	0.62	0.31	Control
A ₁	0	0	+	+	+	+
A ₂	0	0	+	+	+	+
129	0	0	+	+	+	+
133	0	+	+	+	+	+
134	0	0	+	+	+	+
140	0	0	+	+	+	+
146	0	+	+	+	+	+
155	0	0	+	+	+	+
156	0	0	+	+	+	+
161	0	+	+	+	+	+
167	0	0	+	+	+	+
I	0	+	+	+	+	+
IV	0	0	+	+	+	+
V	0	0	+	+	+	+
Staph. aureus	+	+	+	+	+	+
Hem. Strep.	+	+	+	+	+	+

*) In one experiment there was 0 growth of strain V on plates containing 125 units/ml.

On plates containing 10 units/ml was observed reduced growth of staphylococcus aureus, whereas the effect of this concentration on the hemolytic streptococci could not be established with certainty.

Table III.
In vitro sensitivity to sulfathiazole.

Concentration Strain	1/61000 g/ml.	1/128000	1/256000	1/512000	1/1024000	1/2048000	Control
A ₁	0	+	+	+	+	+	+
A ₂	0	0	+	+	+	+	+
129	0	0	+	+	+	+	+
133	0	0	0	+	+	+	+
134	0	0	+	+	+	+	+
140	0	+	+	+	+	+	+
146	0	0	0	+	+	+	+
155	0	0	0	0	+	+	+
156	0	+	+	+	+	+	+
161	0	0	0	+	+	+	+
167	0	+	+	+	+	+	+
I	0	0	0	+	+	+	+
IV	0	0	0	0	+	+	+
V	0	0	0	0	0	+	+
Staph. aureus	+	+	+	+	+	+	+
Hem. Strep.	+	+	+	+	+	+	+

On plates containing 1/500 gr/ml was not yet observed any certain effect on staphylococcus aureus or hemolytic streptococci.

In vivo sensitivity.

Special conditions apply to strain V, and this strain was therefore preferred for the first infection experiment. Incidentally, the real object of the experiment was to prove that the strain was a genuine *Hemophilus Ducreyi* (by producing a typical chancroid) and also to examine the strength of the cutaneous reaction when made with vaccines of homo- and heterologous strains (a report on these investigations will be published at a later date, in a work on the specificity of the cutaneous reaction).

As the infectivity of the strain was not known in advance, a first infection was made with a tiny dose: 0.2 ml intra-dermally on the lower part of the abdomen, of a suspension of 3 colonies in a tube of bouillon (about 7 ml). No trace of infection was observed and infection was then made with a much larger dose: 0.2 ml from a tube of bouillon in which was suspended the culture from a tube of semi-fluid 30 % sheep blood agar (incubated for 4 days at 33° C). An abscess now quickly developed, which perforated spontaneously after 4 days, whereafter the ulceration grew slowly, so that on the 18th day it appeared as an oval lesion with a maximum diameter of 1.5 cms. The chancre was characteristic with marked tenderness, undermined edges and ample secretion of pus.

When the experimental strain V-chancr had existed for 16 days, 100,000 units of penicillin, the Na-salt of G-penicillin, were administered intramuscularly in one dose. Cultivation just before and 24 hours after the injection showed no effect whatever of the injection, as there was confluent growth in both cases. The clinical picture was also quite unchanged. Repeated cultivations in the following days showed unchanged conditions, and when the chancre was 20 days old, local treatment was instituted with penicillin ointment (the Na-salt of G-penicillin, 1,000 units per g). The ointment was changed twice a day, and effect was seen plainly already after two days' treatment, the surrounding reddening and swelling as well as the soreness being on the decline. The penicillin treatment was continued for 4 days, and as the lesion was pure with fresh granulations, the treatment was discontinued. The chancre now healed slowly, and about 18 days later it was completely cured.

The second volunteer was infected with strain I (which was selected in order to give it a »freshening-up« by passage on a human being). An injection on the abdomen was administered corresponding to that which produced infection in the V-strain experiment. The infection developed exactly like the V-strain chancre and perforated spontaneously on the 4th day. Cultivation on plates from chancre pus showed pure culture of strain I, and when the ulcer had existed for 9 days and still showed progression in that period, treatment with penicillin was instituted. For this purpose »Leo-penicillin Retard« was used (a suspension of procain-penicillin-G in sesamoil equal to

300,000 international units/ml, produced by the »Leo« factory in Denmark). 1 ml a day was administered intramuscularly, by which a therapeutic penicillin-concentration in the blood is alleged to be attained for 18 out of 24 hours. Once a day — just before the daily injection — cultivation on plates from pus was made. 4 injections in all were administered (1,200,000 units), and already after the second injection (3rd day of treatment) the effect could be clearly observed, as swelling, reddening and soreness were declining. After the fourth injection the lesion was dry and clean with fresh granulations, and the treatment was then discontinued.

According to the results of the cultivations there was a clear effect already after the first injection, as the number of colonies decreased from several hundred to very few on each plate. After the third injection one plate showed 0 growth and one plate 2 colonies. After the fourth injection both plates showed 0 growth.

Discussion.

The results of these in vitro investigations show — contrary to previous observations — that there are differences between the sensitivity of the strains. This fact is most conspicuous in the sulfathiazole experiments, but less obvious as regards streptomycin. This difference might not be a real one, but might be ascribed to differences in the capacity to grow on the solid medium. On the whole the strains 155 and IV do not grow quite so well, but strain V grows better than any of the other strains. Hence it is a justifiable conclusion that there is a real difference in the in vitro sensitivity, which will probably also be found in vivo.

Furthermore, the tables indicate that the strains will grow at considerably higher concentrations of chemotherapeutics than those found by the mentioned authors. This is probably connected with the fact that they used cultivation methods that did not afford optimal conditions of growth. *Tung* thus cultivated in fluid medium which will give substantially less growth than the solid medium, and *Mortara* used horse blood, which in systematic cultivation investigations with measurements of sizes and numbers of colonies has proved to produce poorer growth than sheep blood (which will give the same growth as rabbit blood). In addition, both authors have incubated for too short times; the growth in fluid media does not reach maximum until after 4—6 days' incubation according to the composition of the medium; and in the case of cultivation on solid medium, the growth will not reach its maximum until after 3 days' incubation.

The investigations also show that penicillin cannot be used for selective media for isolation of Ducrey's bacillus — as stated by *Vilanova* — as the latter will also be inhibited by the doses required to inhibit contaminating staphylococci.

As already mentioned, the Ducrey strains were cultivated under

optimal conditions of growth, which, among other things, means that incubation took place at 33—34° C. In one penicillin experiment it was also tried to cultivate at body temperature 37° C; in that experiment there was growth of all strains on plates containing 0.02 units of penicillin/ml, whereas only the strains A₁, 133, and 156 grew at the concentration 0.04 units/ml. The control test was made at 33° C and showed the same sensitivity as shown in Table I.

For determination of the in vitro sensitivity it will, of course, be necessary to cultivate under optimal conditions for the microbe concerned, even if that will render it difficult — as it does in this case — to make comparisons with the usual test strains. It is found that the figures for their sensitivity are decreasing according as incubation is made for 1, 2, or 3 days, and also that the amount of inoculum is of some importance. The values indicated in the tables for the sensitivity of the test strains apply to three days' incubation, and are consequently misleading. It should also be noted that these values only occur when ample inoculation is given, and they are not constant. If test strains are to be used at all for experiments where the bacteria under comparison require so long incubation, the test strains must undoubtedly be incubated only for the usual 18—24 hours. At this period of growth is found total inhibition of staphylococcus aureus at 0.08—0.16 units of penicillin, about 2.5 units of streptomycin and > 1/500 g sulfathiazole per ml of medium. For hemolytic streptococci the figures are 0.02, 2.5—5.0 and > 1/500, respectively. If these figures are used as a basis for comparison it will be seen that the penicillin sensitivity of *Hemophilus Ducreyi* is the same as or slightly lower than that of staphylococcus aureus, and also that it is substantially lower than that of hemolytic streptococci. In regard to streptomycin it will be seen that the sensitivity is equal to or greater than that of the test strains. The high percentage of blood in the media render the absolute figures for the sulfathiazole concentrations quite insignificant, but the experiments show that the sensitivity is much greater than that of the test strains.

These facts render it impossible to draw any certain conclusions by means of comparisons with the test strains, as to the in vivo sensitivity. Hence, the latter will have to be determined by treatment of experimentally induced chancroid in test animals or volunteers, because naturally occurring chancres are generally so infected with other microbes that a fairly large material will be required to make a reasonably accurate determination of the in vivo sensitivity. Mortara *et al.* (1947) tried to treat chancroid induced in rabbits and found the method satisfactory; it must be pointed out, however, that these chancres showed a marked tendency towards spontaneous healing — Mortara's untreated rabbits recuperated after only a week's time. I have observed the same phenomenon in an experiment where a monkey (*macaca sylvanus*) developed a fine chancroidal ulcer, which, how-

ever, healed without treatment of any kind after about 3 weeks, after having been on the decline for the last 8—10 days.

This tendency in the test animals towards spontaneous healing was my reason for preferring persons who volunteered for the experiments, even if that makes the material so small that it is only possible to form an estimate of the *in vivo* sensitivity. In this connection it must be noted that the experimental chaneres contain many more bacteria than those occurring naturally, and this fact may be of some significance because the effect of penicillin must presumably be dependent to some extent on the number of bacteria in the chaneres.

The results of these *in vivo* investigations must be that the determined values of the *in vitro* sensitivity are equal to or perhaps slightly lower than the *in vivo* values, and it must also be expected that infections with any of the examined 14 strains can be treated with penicillin — with the possible exception of chaneroid infections mixed with penicillinase-producing bacteria.

As stated in the introduction, the doses used by *Percyra et al.* for treatment of their test persons were very small, whereas *Day* could cure his patient with a total of 100,000 units. Hence, the results found in this work correspond to the facts available in literature.

Clinically, the treatment with penicillin or streptomycin will only be of interest in the comparatively rare cases where the patients are hyper-sensitive to sulfonamides, because the risk of overlooking a mixed infection with *spirochaete pallida* will cause the sulfonamides to be preferred.

Judging from the *in vitro* sensitivity to sulfathiazole, the proper clinical therapy of chaneroid must be local treatment with sulfonamides in uncomplicated cases — and several of the mentioned authors have also had good results with this method of treatment — and peroral treatment in cases complicated with bunoes. Chaneroids failing to heal after peroral treatment with the doses usually administered, will often be contaminated by sulfa-resistant microbes, and in such cases better results may be achieved by local treatment with antiseptics than by renewed sulfa-medication, possibly combined with fever treatment.

Summary.

The *in vitro* sensitivity of 14 *Hemophilus Ducreyi* strains to penicillin, streptomycin and sulfathiazole was examined by cultivation on solid medium under optimal conditions of growth: agar with 1 % peptone and 30 % defibrinated sheep blood in Petri dishes which were incubated at 33° C for 3 days in saturated water vapours.

The investigations indicate clearly that there is a very considerable difference in the sensitivity of the strains, and that it is rather smaller than stated by previous authors.

The penicillin sensitivity of the strains is somewhat lower or equal to that of staphylococcus aureus.

Practically speaking the streptomycin sensitivity is equal to that of staphylococcus aureus.

In regard to sulfathiazole the sensitivity is substantially greater than those of staphylococcus aureus and streptococcus hemolyticus.

In order to compare the in vitro with the in vivo sensitivity two volunteers were infected and both developed characteristic chancroids. The first person was cured after 4 days' treatment with penicillin ointment, and the second after intramuscular injection of 300,000 units of »Leopenicillin Retard« every day for 4 days. Thus good conformity was observed between in vitro and in vivo sensitivity.

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PARABIOSIS AND RESISTANCE TO TRANS- PLANTATION

III. STUDIES ON THE RESISTANCE TO TRANSMISSIBLE LEUKEMIA IN MICE BY MEANS OF HETEROGENETIC AND HOMOGENETIC PARABIOSIS.

By *Jorgen Bichel* and *Ib Holm-Jensen*.

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If a rat has been made temporarily susceptible to transplantation of a mouse tumour by means of all over irradiation with X-rays, the parabiotic union of the animal with a non-irradiated partner will make this susceptibility disappear. (Bichel and Holm-Jensen, 1947, 1948).

This fact indicates that the induced resistance to heterotransplantation is, at least partially, governed by humoral factors.

It is generally supposed, that the degree of resistance to tumour transplantation depends upon the genetic difference between the tumour and the animal into which it is transplanted. Consequently, the resistance to transplantation from one species to another should not be qualitatively different from the resistance to transplantation of a mouse tumour from one inbred strain of mice into a mouse from another strain.

According to this view the words »primary« and »secondary« resistance should not be used to designate natural resistance as opposed to resistance induced by active immunization. A mouse tumour inoculated into a rat will grow for a short time, but as it simultaneously evokes an anti mouse-tissue immunity, it will finally be absorbed, as a rule, before it has grown to a palpable size. So the rat cannot be considered primarily resistant. (Compare Bichel and Holm-Jensen, 1947, 1948).

It is generally with some hesitation that the actively induced tu-

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tumour immunity is compared with the immunity resulting from bacterial infections, the principal reason being that tumour immunity seems to be dependent upon the inoculation of living cells. The passive transference of tumour immunity by means of blood and serum has been unsuccessful in most experiments, even if immunity in some cases may be accomplished by implantation of whole organs, (cf. Potter, Taylor and McDowell, (1938), who succeeded in transferring immunity against a mouse leukemia passively, transplanting spleen or liver from actively immunized to susceptible mice). It is, however, very likely that the amount of the antibodies which was transferred in such transmission experiments might have been insufficient, so that the dilution in the recipient may be one of the causes of failure to demonstrate the passive immunity.

As parabiotic union should be a very good way of securing a constant exchange of antibodies between two animals, this method has been used in the following experiments, the aim of which has been to investigate the passive immunization to the transplantation of leukemia in mice.

The method of parabiosis has previously been used by several investigators in the study of immunity to tumours. For a survey we may refer to our earlier communications and to papers by Furth, Barnes and Brower (1940) and by Harris (1943). It appears from these papers that the experimental results have been far from uniform, and very often contradictory, as some investigators have found that tumour immunity *will* be transferred between parabiotic animals, and others that it *will not*. It has even been found, that resistance of a mouse to tumour transplantation in some cases may be broken down by the parabiotic union of the animal with a susceptible partner. (Cloudman, 1943).

The results of the experiments reported below seem to indicate at least one factor, which has not previously been taken into consideration, and which may account for the discrepancy of previous reports.

Material and methods.

The mice used in the present experiments were:

1. *Furth strain Aka*. This strain has been inbred in U. S. A. since 1928 and has been kept in Denmark since 1936. It is a well-known strain with a high incidence of leukemia, as a rule of the lymphatic type.
2. *Rf. strain*. This strain has been inbred for a number of years in U. S. A., and was in 1947 by the courtesy of Dr. Furth presented to the Aarhus laboratories, where the inbreeding has been continued. The incidence of leukemia in this strain is low.
3. *Do-Za strain* (*Dobrowolskaja-Zawadskaja*) inbred in France, kept in the laboratories since 1943. It is a strain with moderate incidence of mammary cancer.

The tumour material has been:

1. Leukemic tumours originating in strain Aka, which for a has been transplanted to Aka mice by subcutaneous inoculation
2. B-leukemia (Krebs mouse leukosis), which has been described in the literature and in our communications. This leukemia is very virulent and "takes" 100 per centage of mice of a mixed stock.

When Aka leukemia is transplanted to Aka mice, one hundred per cent will occur. A tumour will develop at the site of the subcutaneous inoculation and reach palpable size in 5 or 6 days; it then grows progressively until the animals in two to three weeks. When inoculated into Do-Za mice, leukemia does not take at all. The Aka mice are partially susceptible to B-leukemia. Following subcutaneous inoculation a nodule of tumor develops in the course of 5 or 6 days, and then regresses. Sometimes the tumor may be rather small and difficult to palpate. In such instances, however, the animals have proved totally resistant on retransplantation.

In Do-Za mice, however, the B-leukemia grows readily and in 100 per cent kills the animals even though it has occasionally happened that the tumor has regressed.

In Rf. mice implantation of the B-leukemia will take tempo until it develops to about the size of a hazel-nut whereafter it regresses.

The technique used for the parabiotic union was that of *Bunster* with slight modifications as previously reported. The partners were of the same sex and when possible litter mates.

In the following description of the experiments the mice from the same and different strains are designated as *heterogenetic* respectively.

EXPERIMENTS I.

I. *Homogenetic parabioses.*

1. 5 Aka mice were inoculated with B-leukemia. When the tumor takes had disappeared the mice were parabiosed with 5 C3H mice which were inoculated with the same leukemia 2 days after the operation. In four of them no palpable takes occurred, in one (182) a small infiltration was felt for one day. (Exp. nos. 179, 180, 181, 184).

2. In the next experiment, also comprising 5 pairs, the parabiotic unions were established before inoculation, which was made with B-leukemia 7 to 19 days after the operation. When the tumours had regressed the uninoculated parabions were inoculated, but no takes occurred. (Exp. nos. 139, 141, 194, 197, 198).

3. As the Aka leukemia does not take in the Do-Za strain, and the B-leukemia almost invariably kills the animals, only a few experiments with homogenetic Do-Za parabioses have been successful. Only in one single case of the Do-Za Do-Za pairs in which one of the parabions was inoculated *after* the operation, did it happen that a tumour regressed. On subsequent inoculation the partner proved resistant. (Exp. no. 204).

Two Do-Za mice in which tumours of B-leukemia had regressed were parabiosed with non-inoculated Do-Za mice, which on subsequent inoculation proved resistant in one pair (169) while a small temporary take was observed in the other (163).

II. *Heterogenetic parabioses.*

A number of experiments were carried out with pairs consisting of an Aka mouse in parabiosis with a Do-Za mouse. As the Do-Za mice are resistant to the Aka-leukosis, it was considered of interest to investigate whether the parabiotic union of an Aka mouse with a Do-Za mouse would modify the course of the leukemia in the Aka mouse.

1. Aka mice were inoculated with Aka-leukemia 8—15 days before (Experiments nos. 45, 46, 47, 75, 76, 77) or 3—6 days after (Exp. nos. 52, 53, 78, 79, 94, 95) parabiosis with non-treated Do-Za mice. In every case the Aka-leukemia took the ordinary course and progressed uninfluenced by the parabiosis to the death of the animals. None of the Do-Za mice showed any evidence of leukemia.

2. In some experiments of this type the Do-Za mice were inoculated with Aka-leukemia before the union with the Aka-mice, but even then the course of the leukemia in the Aka mice was unaffected. (Exp. nos. 84, 85, 86, 87, 73, 74).

3. In still other experiments the Do-Za mice were inoculated after the parabiosis and the Aka mice were not. (Exp. nos. 98, 99, 101). No leukemia developed in the Aka mice, indicating, that few if any leukemic cells passed the parabiotic junction in these cases.

The following experiments were carried out with B-leukemia, to which the Aka mice are partially resistant, but which in the Do-Za mice generally grew progressively and killed the animals.

4. Aka mice were inoculated with B-leukemia 20 days before (Exp. nos. 172, 174, 175, 176, 177, 178, 109, 112, 113) or 6 to 7 days after parabiosis with non-treated Do-Za mice. All mice developed the typical temporary take. On the subsequent inoculation of the B-leukemia into the Do-Za mice huge tumours developed in all cases causing the death of the animals.

Discussion.

In these experiments the resistance to the transplantation of the applied leukemias was in no cases transferred from one parabion to the other in the heterogenetic parabioses. The experiments with the homogenetic parabioses gave, however, indication of a transmission of resistance against the B-leukemia to which the Aka mice are only

partially resistant. *On this basis we advance the hypothesis that the transmission of the induced resistance to transplantation of leukemias between parabiotic mice is favoured by homogenetic and inhibited by heterogenetic parabioses.* It is, of course, not justified to generalize from those few experiments, and such a hypothesis is in reality only valid for the leukemias and animal stocks for which it has been proved by experiment. The combination of mice strains and tumour material in the above mentioned experiments is not too ideal either, as the Aka mice were rather too resistant, and the Do-Za mice too susceptible to the B-leukemia. To further investigate the validity of the advanced hypothesis a series of experiments was carried out with transplantation of B-leukemia to homogenetic parabioses of Rf. mice. As stated in the description of the animal material, the Rf. mice are partially susceptible to the B-leukemia in which respect they hold a medium position between the Do-Za and the Aka mice.

EXPERIMENTS II.

1. This experiment consisted of 4 Rf. mice which were inoculated with B-leukemia. The typical temporary takes occurred and 43 days later the animals were united in parabiosis with non-treated Rf. mice. Seven days after the operation the new mice were inoculated and contrary to our expectations all of them developed tumours. (Exp. nos. 276, 277, 279, 281). As it was felt, that the interval between immunization and parabiosis might have been too long, this interval was diminished in the following experiments:

2. 17 Rf. mice were inoculated with B-leukemia 14 to 19 days before parabiotic union with non-treated Rf. mice. The temporary take had disappeared before the operation in all cases. Five or six days after the establishment of parabiosis the new mice were inoculated with B-leukemia, which resulted in the development of a small tumour in two animals, an uncertain take in one animal and no takes at all in the 14 other mice. (Exp. nos. 315, 316, 317, 337, 338, 341, 343, 344, 345, 346, 347, 350, 351, 352, 354, 357).

3. In 3 supplementary experiments the Rf. mice were not immunized before the parabiosis, but B-leukemia was transplanted to one of the parabions after the operation. A typical temporary take occurred, indicating that the parabiosis *per se* does not influence the susceptibility. The non-treated partner in these experiments was later inoculated with B-leukemia and proved resistant in all 3 pairs. (Exp. nos. 297, 299, 300).

Conclusion and Comments.

The above experiments clearly demonstrate that the induced resistance to a transplantable leukemia in mice may be transferred to other mice of the same strain by parabiotic union of the animals. In

experiment II. 2. complete resistance was conferred on the unimmunized partner in 14 out of 17 experimental pairs and increased resistance in the three remaining pairs. The results probably also show that a large amount of antibodies must be transferred in order to secure immunity, as complete resistance was not attained in all cases despite the very good possibility of a constant exchange of antibodies which presumably prevails in parabiosis. From this evidence it is no wonder that passive immunization by means of serum and blood has been unsuccessful.

While our experiments prove that induced resistance to leukemia may be transferred from one animal to another in parabiosis of homogenetic pairs it appears from the experiments described above under I and II, that such a transmission was never found in heterogenetic parabioses. As the possibility exists that the exchange of antibodies should be great to confer total resistance, even on a partially susceptible animal, it seems probable that an insufficient exchange of blood may be responsible for the failure of the transference of immunity in the heterogenetic parabioses.

To investigate this point we carried out determinations by means of labelled red blood corpuscles of the rate of exchange of blood between homogenetic and heterogenetic parabioses (Bichel and Holm-Jensen, 1949). These experiments demonstrated the crossed blood flow to be of the order of 100 times as large in homogenetic as in heterogenetic parabioses. Taken together with the immunity experiments these findings indicate *that immunity to transplantable leukemia may be transferred between mice in parabioses when the exchange of blood is large enough. This may be the case in homogenetic parabioses, whereas it has never been demonstrated in heterogenetic parabioses.*

If this hypothesis is applied to earlier findings of other investigators, the apparent discrepancy of their results will disappear.

Furth, Barnes and Brower (1940) found that the development of leukemia in a susceptible mouse inoculated with leukemia was not influenced by its parabiotic union with a resistant mouse. And they concluded that it was unlikely that inherited resistance and susceptibility are governed by humoral factors. Their experiments were partially of the same type as those described in the present paper, comprising heterogenetic parabioses.

Harris (1943) united mice in parabioses and inoculated one parabion with tumours known to induce immunity in the hosts. Whether the tumours grew progressively or whether, after some time, they regressed, the non-inoculated parabion was found to be immune against the same tumour ten days after the transplantation. In these experiments inbred strains and homogenetic pairs were used, in which according to our hypothesis the immunity should be transferred.

It would be of interest to investigate these problems under conditions in which the rate of exchange of blood between the parabiotic

partners is of the same order of magnitude both in heterogenetic and in homogenetic parabioses. Such conditions might be established by means of a regular *circulation croisé*.

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THE INFLUENCE OF COMPLEMENT UPON AGGLUTINATION*)

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It has long since been established that complement is not necessary for the agglutination reaction, and the general conception is that complement does not even influence agglutination. Curiously enough there does not seem to have been any distinct investigation as to the possible rôle of complement upon agglutination. True enough some investigations concerning the influence of normal serum upon agglutination have been conducted from time to time. Thus *R. H. Dean* in 1912 (1) and *Harry Eagle* in 1930 (2) found an increasing titer of agglutination on addition of euglobulin to the dilutions of serum. The euglobulin was thought to be absorbed by the bacteria in the presence of immune serum thus influencing their surface, rendering them more liable to cohesion and flocculation. No differentiation between active and inactive serum was made. *P. B. White* (3), however, found that the addition of normal rat- or rabbit serum in the concentration of 1:5—1:20 caused a complete dispersion of bacteria in the *Salmonella* group. In 1934 *J. T. Duncan* (4) made a thorough investigation of the influence of normal serum upon agglutination. He found a slight retardation of the rapidity of agglutination when normal serum was added to a mixture of antigen and antibody, when these two components occurred in optimal proportions. When, however, the immune serum was present in large amounts, normale serum added to the reagents had a promoting effect upon the rapidity of the reaction. The increased rapidity was especially recognizable when large amounts of antibodies caused a prezone effect. Duncan, however, did not differentiate between active and inactive sera either.

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OWN INVESTIGATIONS.

Starting upon an entirely different problem, we happened to put up some agglutination tests between a strain of Flexner bacilli and its corresponding immune serum, and to this test we added fresh guinea pig serum in dilution 1:10 with a corresponding series of tubes to which were added the same guinea pig serum in inactivated condition. This experiment was as follows:

Experiment I.

Antigen: Living Flexner bacilli, emulsion 1000 mill. pr. ml.

Antibody: Homologous serum, dilutions 1:200—1:6400. Active guinea pig serum, 1:10.

Normal serum: Guinea pig serum inactivated at 56° C. ½ hour.

Reading of results after incubation at 37° C. for 2 hours.

A.

Antigen and Immune serum alone. 0.5 ml. amounts of each

Dilutions of immune serum:					
1:200	1:400	1:800	1:1600	1:3200	1:6400
Agglutination:					
+++	+++	++	+	+÷	÷

B.

Experiment as A with the addition of active guinea pig serum:

1:10	1:10	1:10	1:10	1:10	1:10
Agglutination:					
÷	÷	÷	÷	÷	÷

C.

Experiment as A with the addition of inactivated guinea pig serum:

1:10	1:10	1:10	1:10	1:10	1:10
Agglutination:					
+++	+++	+++	+	+÷	÷

Conclusion:

Active guinea pig serum inhibited agglutination, while the same serum in inactivated condition showed no effect upon the reaction.

Experiment II.

In this experiment the same antigen, the same immune serum and the same active guinea pig serum were used, but no heated g. p. serum was employed. However, this time the g. p. serum was titrated, while the immune serum was employed in a fixed dose, i. e. 1:200.

B.

All reagents as in A.

ve g. p. serum:									
l. after 8 hours:	+	÷	÷	÷	÷	÷	++	+++	+++
l. after 20 hours:	+	÷	÷	÷	++	+++	+++	+++	+++
Active g. p. serum:									
l. after 8 hours:	+++	+++	+++	+++	+++	+++	+++	+++	+++
l. after 20 hours:	+++	+++	+++	+++	+++	+++	+++	+++	+++

C.

All reagents as in A and B.

ve g. p. serum:									
l. after 8 hours:	÷	÷	÷	÷	÷	++	+++	+++	+++
l. after 20 hours:	+++	+++	÷	÷	++	+++	+++	+++	+++
Active g. p. serum:									
l. after 8 hours:	+++	+++	+++	+++	+++	+++	+++	+++	+++
l. after 20 hours:	+++	+++	+++	+++	+++	+++	+++	+++	+++

Conclusion:

Active guinea pig serum inhibits completely the agglutination of *S. typhi* in homologous serum in doses 0.125—0.016 while the same serum in heated condition does not show any inhibition. No difference is seen whether all factors are mixed simultaneously or after antigen and immune serum separately have been in contact with guinea pig serum before the third factor is added.

Experiment IV.

Consequently it seems justified to assume that Exp. I, II and III show an inhibiting action of active guinea pig serum upon the agglutination of the O antigens of *Shigella* and *S. typhi*. To find if this was also the case with the H antigens, Exp. IV was made with *S. typhi* H and corresponding immune serum. This experiment was connected with a hemolytic test, which was made after the reading of the agglutination test and in the same emulsions as the latter, the aim being to find out whether the complement present in the active guinea pig serum was exhausted in the agglutination reaction, or still present. A control test showing the complement function of the guinea pig serum used was made simultaneously with the test proper.

A. Active guinea pig serum:

0.25	0.125	0.063	0.032	0.016	0.008	0.004	0.002	0.001
Immune serum of <i>S. typhi</i> H. Dilution 1:160. Amount 0.5 ml.								
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Agglutination:

÷	÷	÷	÷	÷	÷	+	++	+++
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B. Control with inactivated g. p. serum:

+++	+++	+++	+++	+++	+++	+++	+++	+++
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Hemolysis after addition of hemolytic system to Exp. A.:

0	0	0	0	0	0	0	0	0
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Hemolysis in control test:

100	100	100	100	100	75	0	0	0
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100 — complete hemolysis. 75 — 75 % of hemolysis. 0 — no hemolysis.

Conclusion:

Experiment IV shows that also in tests with *S. typhi* H the same inhibiting effect of the addition of active guinea pig serum is found as in *Shigella*- and *S. typhi* O experiments. The experiment also demonstrates that the factor showing this inhibiting effect seems to be identical with or to be closely related to the function called complement in guinea pig serum, as the titers of the inhibiting effect and the hemolytic effect are the same, and moreover the hemolytic effect of the guinea pig serum is completely exhausted by the inhibiting function.

While it seems natural to conclude that the inhibiting function really is caused by the factor called complement, this assumption is not verified by the fact that the titers of both functions seem to be alike in the same active g. p. serum. We therefore sought to bring forth more facts about this relation. First we made an experiment with human sera both as to the inhibiting function and as to the usual complement function in hemolytic tests. This experiment is as follows:

Experiment V.

Three active human sera from different persons were titrated in double series of test tubes from 0.25 until 0.004 ml. To one series was added *S. typhi* O antigen with its corresponding immune serum in dilution 1:160. Reading of this series was made after incubation for 4 hours. To the other series of each human serum a hemolytic system was added, and the hemolytic titer read after ordinary incubation.

Human sera titrated as follows:

	0.25	0.125	0.063	0.032	0.016	0.008	0.004
Serum I							
Agglutination	÷	÷	÷	÷	+	++	+++
Hemolysis	100	100	100	100	0	0	0
Serum II							
Agglutination	÷	÷	÷	÷	÷	÷	+
Hemolysis	100	100	100	100	100	trace	0
Serum III							
Agglutination	÷	+	++	+++	+++	+++	+++
Hemolysis	100	0	0	0	0	0	0

Conclusion:

Exp. V shows the same correlation between inhibition and hemolytic power of active human sera as that found in active guinea pig sera. The poor inhibiting and hemolytic functions of serum III are especially noteworthy.

Consequently Experiments IV and V seem to justify a strong suspicion that the inhibiting factor and the usual complement really is the same individual factor. To find additional evidence in support of this view we tried different fractions of the active g. p. serum after treatment with $(\text{NH}_4)_2\text{SO}_4$.

Experiment VI:

Antigen: *S. typhi* O. Immune serum: *S. typhi* O, dilution 1:50 G. P. serum or fraction.

Serum or fraction	0.5	0.25	0.125	0.063	0.032	0.016	0.008
Active serum untreated	+++	+	÷	÷	+	+++	+++
1/3 saturation	++	+	÷	++	+++	+++	+++
1/2 »	+++	+++	+++	+++	+++	+++	+++

Technique and reading as in Exp. V.

Exp. VI shows that the inhibiting factor disappears from the active g. p. serum after saturation with ammonium sulphate to $\frac{1}{2}$ volume while $\frac{1}{3}$ saturation leaves some function in the treated serum.

Experiment III and VI show that large doses of g. p. serum may give some agglutination, or rather show a loss of inhibiting function as compared with smaller doses of the same serum. This fact may be caused by some power of unspecific agglutination on the part of the g. p. serum. This initial lack of inhibition is seen in many tests, when the larger doses of g. p. serum are employed. To ascertain this fact we made an experiment to elucidate this point.

Experiment VII:

Antigens: *S. typhi* H and O, *S. paratyphi* B, H and O.

Serum: Active g. p. serum.

Technique as in the foregoing tests.

	G. P. serum in doses:						
	0.5	0.25	0.125	0.063	0.032	0.016	0.008 ml
<i>S. typhi</i> O	++	+	÷	÷	÷	÷	÷
<i>S. typhi</i> H	÷	÷	÷	÷	÷	÷	÷
<i>S. paratyphi</i> B O.	+	÷	÷	÷	÷	÷	÷
<i>S. paratyphi</i> B H.	÷	÷	÷	÷	÷	÷	÷

Conclusion:

Exp. VII shows a distinct agglutinating effect of active guinea pig serum on *S. typhi* O and *S. paratyphi* O, but not on the H antigen of the same microbes. The initial lack of inhibiting effect of g. p. serum in the former tests then seems to be explainable as an unspecific agglutinating effect of g. p. serum in high doses.

According to the experiments already shown it seems justified to conclude that complement is able to inhibit the agglutination of different antigens by their homologous immune sera. It remains to be seen whether there may be some correlation between the doses of complement and the doses of immune serum. This was the aim of experiment VIII.

Experiment VIII:

Antigen: *S. typhi* O. Immune serum *S. typhi* O and OH.
Technique as before.

		<i>A. Immune serum O.</i>							
		Complement in doses:							
Im- mune serum		0,25	0,125	0,063	0,032	0,016	0,008	0,004	0,002
1:10	Incub.	++	++	÷	÷	÷	+++	+++	+++
1:100	8 hours	+++	+++	÷	÷	÷	÷	÷	+
		<i>B. Immune serum OH.</i>							
1:10	Incub.	++	++	÷	÷	÷	++	++	++
1:100	8 hours	++	++	÷	÷	÷	++	++	++
1:1000		++	++	÷	÷	÷	÷	÷	++
1:10	Incub.	+++	+++	+++	+++	+++	+++	+++	+++
1:100	20 hours	+++	+++	+++	+++	÷	++	++	+++
1:1000		+++	+++	+++	+++	÷	+	++	++

Agglutinating titers: Serum O 1/640. Serum OH 1/2560.

Conclusion:

This experiment, which has been repeated with the same results, seems to indicate that there exists a certain correlation between amounts of immune serum and doses of complement, in as much as the titer of inhibition of complement increases with diminishing amount of immune serum. It also shows that a long period of incubation seems to be able to counteract inhibition.

Discussion.

The experiments dealt with in this paper make it clear that a factor resident in active guinea pig serum is able to inhibit the agglutination between *Shigella* and *Salmonella* strains and their respective homologous sera. It seems further that this factor is either the complement or a factor with the biological characters of the latter. No such inhibition of agglutination is found in inactivated guineapig serum.

Several facts seem to substantiate the hypothesis that the said factor really is complement: the corresponding titers of inhibition and hemolytic power of individual specimens of guinea pig serum and the exhaustion of inhibiting as well as of hemolytic activity after treatment of the guinea pig serum with increasing doses of ammonium sulphate. Further, no trace of hemolytic activity remains in mixtures of bacteria, immune sera and active guinea pig serum, when those mixtures after the inhibition has occurred are added to a hemolytic system, showing that the complement is either fixed or destroyed during the previous reaction.

We may consequently surmise that complement has been adsorbed

to the antigen-antibody compound formed during the incubation period and that this absorption inhibits the agglutination. The experiment may consequently be regarded as a simple complement fixation test. But the problem to solve will then be this: How can complement interfere with the normal agglutination between the two normal partners in an agglutination reaction? It will be remembered that according to modern conception (Marrac and Heidelberger *et al.*) the antibody is a bivalent factor able to bind two antigens to itself, while the antigen is a multivalent factor being able to attach itself to several molecules of antibodies. When these two factors react in ideal proportions a lattice is formed, and this lattice is able to grow into large compounds, ultimately growing so large that they precipitate when one is dealing with soluble antigens, and agglutinate when the antigens are solid.

If then some factor interferes with the fixation of antibody to antigen or inhibits the fixation of individual antigen-antibody compounds to neighbor compounds of the same nature, no agglutination can occur, since no large clumps of antigen-antibody compounds can be formed. This fatal occurrence may be caused in three ways, when we disregard the occurrence of half-haptenes and only consider the possible action of complement. First, the complement might be thought to attach itself to the antibody end of the compound, secondly, it might attach itself to the antigen end, or, third, it might be thought to attach itself to the antigen-antibody compound as a whole.

Theoretically all three hypotheses may meet the facts, but it is rather improbable that the two first hypotheses should be correct, since it is well known that complement in ordinary complement fixation tests is never fixed by any of the two first mentioned factors in their isolated condition. It is only when the antigen is sensitized, that complement can be fixed. It seems therefore most probable that complement is adsorbed to the sensitized whole compound of antigen-antibody before the isolated individual compounds have had a chance to combine with neighbor compounds. The biochemical explanation of adsorption is obscure. One might think that the sensibilisation of the antibody calls forth some altered potential in the compound that is able like a magnet to draw the complement to the surface of the compounds. The latter may then be covered with a film of protein inhibiting the formation of the lattice necessary for the agglutination by isolating the individual antigen-antibody compounds from each other.

The inhibition of agglutination by complement is dependent upon certain conditions in the experiment. Thus a very considerable amount of immune serum and a small amount of complement will show no inhibition, evidently because the few molecules of complement present are unable to hinder the formation of a lattice large enough to agglutinate, since only few compounds of antigen-antibodies are blocked by the complement. A prolonged incubation also tends to obliterate

the inhibition. It seems that the tendency on the part of the antigen-antibody compounds to unite is strong enough to penetrate the hindrances of the hypothetical film of complement, if they are only given time enough to do it.

The explanation of the initial agglutination in tests with large doses of complement may be that the normal agglutinins often present in guinea pig sera add their forces to those of the immune serum and thus inhibit the action of the complement.

Conclusions:

1. The agglutination reaction between *Shigella* and *Salmonella* strains and their homologous immune sera may be inhibited by a factor resident in active immune sera.
2. The inhibiting factor has been identified with complement.

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TWO NEW SALMONELLA TYPES FROM THE BELGIAN CONGO

By F. Kauffmann and R. Reul.

(Received for publication December 13th, 1948.)

1. *Salmonella aequatoria*.

Two strains of this type (C 13 and C 15) were isolated in Coquilhatville in 1946 from feces of two European patients with diarrhea, in July and September respectively.

The strains possessed the usual cultural and biochemical attitudes of the *Salmonella* group. No fermentation of adonitol, inositol, lactose-salicin, and sucrose. No production of indole, no liquefaction of gelatin, and no decomposition of urea. Rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol (with gas), rhamnose, sorbitol, trehalose, and xylose. Positive reaction in Stern's glycerol-fuchsin broth, and formation of H_2S . Prompt growth on Simmons' agar containing glucose and sodium-citrate. Positive reaction in d-tartrate, l-tartrate, mucate, and sodium-citrate after 1 day, and in i-tartrate after 2—3 days.

Serologically both strains are identical and have the formula VI, VII : $z_4, z_{23} : e, n, z_{15}$.

Cross-absorptions showed that the O antigen VI, VII is identical with the O antigen of *S. thompson* (VI, VII : $k : 1,5$), the H antigen z_4, z_{23} with the H antigen of *S. cerro* (XVIII : $z_4, z_{23} : -$), and the H antigen e, n, z_{15} with the H antigen of *S. glostrup* phase 2 (VI, VIII : $z_{10} : e, n, z_{15}$).

As all other *Salmonella* types with the H antigenic complex $z_4 \dots$ are monophasic, this is the first diphasic type with H antigen z_4, z_{23} showing $\alpha - \beta$ -phase variation.

2. *Salmonella mbandaka*.

This culture (Ngubela) was isolated in Coquilhatville on May 7, 1948 from the feces of a negro with typhoid fever. On April 23rd a strain of *Salmonella typhi* was isolated from the patient's blood.

The cultural and biochemical behaviour of *S. mbandaka* is identical with the above described behaviour of *S. aequatoria*.

Serologically the strain has the formula VI, VII : $z_{10} : e, n, z_{15}$. Cross-absorptions showed that the O antigen VI, VII is identical with the O antigen of *S. thompson* and that the H antigen $z_{10} : e, n, z_{15}$ is identical with the H antigen of *S. glostrup* (VI, VIII : $z_{10} : e, n, z_{15}$).

Summary.

The authors describe two new *Salmonella* types from the Belgian Congo: *S. aequatoria* = VI, VII : $z_4, z_{23} : e, n, z_{15}$ and *S. mbandaka* = VI, VII : $z_{10} : e, n, z_{15}$.

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TWO NEW SALMONELLA TYPES FROM THE BELGIAN CONGO

By F. Kauffmann, E. van Oye and J. Vandepitte.

(Received for publication December 13th, 1948.)

1. *Salmonella makiso*.

This culture (strain C. 1122) was isolated in 1948 from feces of a 3 years old native boy with enteritis, in Stanleyville.

The cultural and biochemical behaviour of *S. makiso* is typical for members of the *Salmonella* group. No fermentation of adonitol, inositol, lactose, salicin, and sucrose. No production of indole, no liquefaction of gelatin, and no decomposition of urea. Rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol (with gas), rhamnose, sorbitol, trehalose, and xylose. Positive reaction in Stern's glycerolfuchsin broth, and formation of H_2S . Prompt growth on Simmons' agar containing glucose and sodium-citrate. Positive reaction in d-tartrate, l-tartrate, mucate, and sodium-citrate; negative reaction in i-tartrate.

Serologically the strain has the formula VI, VII : 1, $z_{28} : z_6$. Cross-absorptions showed that O antigen VI, VII is identical with the O antigen of *S. thompson* (VI, VII : k : 1.5). The H antigen 1, z_{28} is almost identical with the H antigen of *S. javiana* phase 1 (I, IX, XII : 1, $z_{28} : 1.5$), as H serum of *S. javiana* phase 1 was completely exhausted by *S. makiso* phase 1, but in H serum of *S. makiso* phase 1 (titer 1 : 3200) H agglutinins with a titer of 1 : 100 for *S. makiso* phase 1 remained after absorption with *S. javiana* phase 1. H antigen z_6 of *S. makiso* is identical with H antigen of *S. kentucky* = (VIII), XX : i : z_6 .

2. *Salmonella kibnsi*.

This culture (strain C 590) was isolated in 1948 from feces of a native soldier with typhoid-like infection, in Stanleyville.

The cultural and biochemical behaviour of *S. kibusi* is identical with the above described behaviour of *S. makiso*.

Serologically the strain has the formula XXVIII : r : e, n, x.

Cross-absorptions showed that the O antigen XXVIII is identical with the O antigen of *S. tel-aviv* (XXVIII : y : e, n, z_{15}), that H antigen r is identical with H antigen of *S. virchow* phase 1 (VI, VII : r : 1, 2), and that H antigen e, n, x is identical with H antigen of *S. abortus equi* (IV, XII : — : e, n, x).

Summary.

The authors describe two new *Salmonella* types from the Belgian Congo *S. makiso* = VI, VII : l, z_{28} : z_6 and *S. kibusi* = XXVIII : r : e, n, x.

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F. Kauffmann: Die Bakteriologie der Salmonella-Gruppe, Einar Munksgaard, Copenhagen, 1941.

A CASE OF POLYAGGLUTINABLE HUMAN RED CELLS

By *K. Henningsen.*

(Received for publication December 15th, 1948.)

Since 1938 six cases of polyagglutinable human red cells have been reported (1, 2, 4, 5) the characteristic feature of which was agglutinability by a majority of human sera independently of the different bloodgroupsystems. Of the six cases reported the blood in four originated from patients suffering from serious acute diseases, one from a boy treated for congenital syphilis, and one from a healthy young woman. All of them belonged to group O, and none of them were agglutinated at 37°. By titration and absorption experiments some relationship to the T-receptor of the Hübener-Thomsen-Friedenreich phenomenon was demonstrated (2, 4), and the possibility of a correlation of the corresponding agglutinin to the non-specific cold-agglutinins was discussed. The peculiar property of the cells appeared to be an induced transformation, and it was shown to be transitory in three (four) of the cases.

The author has recently encountered a similar blood specimen. It originated from a four-year-old healthy boy, and the blood was referred to the Institute in a case of disputed paternity. Information obtained from the family's usual practitioner*) revealed that the boy never had suffered from any serious illness or had been treated with any special drugs or sera. The blood, designated blood no. 38, belonged to group OM, the serum reacting strongly with A- and B-blood-cells, the corpuscles, however, being agglutinated to a varying degree by most adult human sera, irrespectively of group. The corpuscles were not agglutinated by their own serum or by the sera of the boy's father or mother, but reacted strongly with the serum of a 3-year-old sister. The tests were all performed on glass slide at room temperature, and the reactions were read macroscopically after 15—20 minutes. The results of those readings are given in table 1.

*) My thanks are due to dr. Budtz-Olsen, Roskilde.

Table 1.

Reaction of cells no. 38 against sera from:

	+	—	total	per cent +
adults:	122	23	145	84
children:	9	40	49	18,4

By absorption it was ascertained that the reactions depended on a specific agglutinin present in most human sera. The red cells of no. 38 removed this agglutinin completely by absorption with one fourth volume of cells at room temperature for 2 hours. The anti-A- and anti-B-agglutinins, however, were not inhibited, just as absorption with A- and B-cells did not affect the specific agglutinin for cells no. 38. (Table 2.)

Table 2.

Serum	abs. with cells:	tested against cells:	serum dilution					
			1/2	1/4	1/8	1/16	1/32	1/64
Anti-A	no. 38	A ₁	+++	+++	+++	+++	++	+
Anti-A	unabs.	A ₁	+++	+++	+++	+++	++	+
Anti-B	no. 38	B	+++	+++	+++	++	+	+
Anti-B	unabs.	B	+++	+++	+++	++	+	+
Anti-A	unabs.	no. 38	++	++	++	+	—	—
Anti-A	no. 38	no. 38	—	—	—	—	—	—
Anti-B	unabs.	no. 38	++	+	+	(+)	—	—
Anti-B	no. 38	no. 38	—	—	—	—	—	—
Anti-A	A ₁	A ₁	(+)	—	—	—	—	—
Anti-A	A ₁	no. 38	++	++	+	(+)	—	—
Anti-B	B	B	—	—	—	—	—	—
Anti-B	B	no. 38	++	+	(+)	—	—	—

Repeated washings with icecold and warm saline did not alter the reactions.

In order to investigate the influence of the temperature, the cells were tested in dwarf tubes against 2 rather potent sera. Prior to testing the sera were absorbed in the refrigerator with half a volume of their own cells in order to avoid the source of error which the non-specific cold-agglutinins constitute. After this absorption agglutination of normal O-cells could not be produced at any temperature. It is remarkable that one of those sera agglutinated cells no. 38 at 37° as contradistinguished from the findings in earlier reported cases. The reactions, however, were definitely stronger at room-temperature and strongest at 4°. The result of the titrations is shown in table 3.

Table 3.

Temperature	Serum	Serum dilution						
		1/1	1/2	1/4	1/8	1/10	1/32	1/64
37°	4/11/11	f. tr.*)	—	—	—	—	—	—
37°	5/11/11	++	+	—	—	—	—	—
21°	4/11/11	++	++	+	(+)	—	—	—
21°	5/11/11	+++	++	++	+	(+)	—	—
4°	4/11/11	+++	+++	+++	+++	+++	++	+
4°	5/11/11	+++	+++	+++	+++	+++	++	+

*) f. tr. = faint trace.

The titer of the specific agglutinin is not diminished significantly by absorption of the non-specific cold-agglutinins nor does absorption of the specific agglutinin diminish the titer of the non-specific cold-agglutinins.

The specific receptor was not encountered in the blood of the boy's family (father, mother, sister), or by testing a number of random blood samples, totalling 65.

In the serum of blood no. 38 anti-A-agglutinin (titer 64) and anti-B-agglutinin (titer 16) were demonstrated. Non-specific cold agglutinins were not demonstrable.

The polyagglutinability was transmissible neither by direct inoculation of a fresh normal suspension with the abnormal suspension nor by inoculating from a dried crust of the abnormal blood. Further a new specimen was obtained which was tested about an hour after the blood was taken. This specimen gave identical reactions. It may thus be taken for granted that the abnormal receptor is present in vivo and that the reaction of the blood is not resulting from bacterial contamination. As it was impossible to obtain the blood specimen sterile, bacterial cultivation from the blood has not been performed.

A few days later the author by a lucky coincidence received a blood specimen, group B N, exhibiting a typical Hübener-Thomsen-Friedenreich-phenomenon. This blood, designated blood W. J., was agglutinable by a majority of human sera independently of group, and the property was transmissible to new normal suspensions. Cells no. 38 and cells transformed by blood W. J. were tested against a number of sera, the results showing a very close correlation. By absorption experiments it was established that the agglutinin reacting with cells no. 38 was identical with the T-agglutinin of Friedenreich.

The identity of the agglutinins was further confirmed by a splitting-off experiment (absorption by cells no. 38 in the refrigerator, releasing of the agglutinin by 48°), by which the specific agglutinin was obtained in saline. This split-off agglutinin reacted strongly with

Table 4.

Serum	abs. with cells	tested against cells	serum dilution				
			1/1	1/2	1/4	1/8	1/16
7/8/11	unabs.	no. 38	++	+	+	—	—
7/8/11	unabs.	transf. by W. J.	++	+	+	—	—
7/8/11	no. 38	no. 38	—	—	—	—	—
7/8/11	no. 38	transf. by W. J.	+	—	—	—	—
7/8/11	transf. W. J.	no. 38	(+)	—	—	—	—
7/8/11	transf. W. J.	transf. by W. J.	(+)	—	—	—	—

cells no. 38 and with bacterially transformed cells. By testing no. 38 serum against cells transformed by blood W. J. the corpuscles were weakly agglutinated by microscopical control, but it must be borne in mind that the cells had been produced by direct bacterial inoculation, and as such were liable to spontaneous agglutination.

According to these findings it might be possible that the serum of blood no. 38 contains an agent which is able to transform the latent T-receptor *in vivo* in accordance with the transforming quality of bacterial filtrates of Friedenreich *in vitro*. In order to obtain evidence with regard to this hypothesis, normal group O cells were suspended in about 5 volumes of serum from blood no. 38 and kept for 24 hours at 37°, room temperature and 4°. After washing, the corpuscles were tested against potent anti-T-sera. None of the cells were agglutinated. On account of scarcity of serum it was impossible to treat more samples in this way.

In order to further investigate the relationship between the polyagglutinable receptor of blood no. 38 and the T-receptor of Friedenreich, an attempt was made of producing panagglutinable cells by treating them with several strains of actinomycetes (R. von Magnus). One of these strains, no. 16, transformed red corpuscles by direct inoculation as well as by treating the cells with centrifugated broth, in which the bacilli had been cultivated. In testing corpuscles transformed by this broth against a number of sera, the cells appeared panagglutinable, but the reactions did not correspond exactly to the reactions of cells no. 38. In accordance with this, absorption experiments revealed that the corresponding, apparently specific agglutinins were not identical. (Table 5).

Discussion.

In the formerly reported cases polyagglutinability appears to be an induced property of the red blood cells disappearing gradually in the course of some months. The supposed transforming agent has not been identified. In five of the cases severe bacterial disease or drugs

Table 5.

Serum	absorbed with cells	tested against	
		cells no. 38	O-cells transf. by actinomyce.
8/11/11	no. 38	—	+++
»saline«*)		+++	—
7/8/11	unabsorbed	+++	++
7/8/11	transf. by W. J.	—	+++
18/22/11	normal O-cells	+++	+
18/22/11	O-cells transf. by actinomyce.	+++	—

*) Split-off saline containing the specific anti-38 agglutinin.

may be suspected of producing the abnormal property, but in Gaffney and Saeh's second case as well as in the case just reported the abnormal blood cells originate from apparently quite healthy individuals. The author has not been able to demonstrate the transforming agent in the serum of the abnormal blood.

The abnormal receptor appears to be identical with a receptor originating from bacterial transformation of blood corpuscles. On the other hand it is demonstrated, that this so-called T-receptor is not a serological entity, blood-cells transformed by a strain of actinomycetes appearing qualitatively different from blood-cells transformed by an accidentally encountered, not identified transmissible agent. Several related but not identical T-receptors might explain the apparent inconsistency of the experiments of Boorman et al.

As mentioned by Boorman et al., it is very remarkable, that this peculiarity has been demonstrated in group O bloods only. It must be taken into consideration, however, that the double set agglutinins of O-blood increases the chances of detecting any discrepancies between cells and serum. It cannot be excluded, however, that O-cells may offer the transforming principles more favourable conditions than A- and B-bloods.

The clinical and forensic importance is slight, the phenomenon being so rare. The possibility of its occurrence, however, must be borne in mind, just as the Hübener-Thomsen-Friedenreich phenomenon, and in testing dubious cells against several AB-sera the abnormal condition of the cells is revealed.

Summary:

1) A case of polyagglutinable human red cells is reported. The anomaly was not transmissible and appeared to have come into existence in vivo.

2) The abnormal receptor is found to be identical with the T-receptor of Friedenreich as produced by an accidental, contaminated blood specimen.

3) By treating normal blood cells with a strain of actinomycetes the cells were made polyagglutinable, but the receptor produced in this way appeared qualitatively different from the one produced by the contaminated specimen, thus indicating the existence of more than one, possibly related T-receptors.

Addendum: 3 months later the red cells of the boy were agglutinated by 52 per cent only of adult human sera. The reactions were definitely weaker consisting of a few agglutinates among numerous free corpuscles, thus indicating the termination of the survival of the transformed cells.

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AN INVESTIGATION OF THE BIOCHEMICAL REACTIONS OF HEMOPHILUS DUCREYI

By Flemming Reymann.

(Received for publication December 31st, 1948.)

In consequence of the difficulties in isolating and keeping Ducrey's bacilli, only scattered and brief reports have so far been published of the bacteriology of the microbe. As a continuation of previous works on (1) Conditions of Growth, (2) Diagnostic Cultivation, and (3) Sensitivity to Chemotherapeutics, this work aims at elucidating the biochemical reactions of the Ducrey bacillus.

A number of authors have investigated the fermentation reactions of the bacillus, and have arrived at widely different results: Kamimura, Y. and K. Abe (1912) discovered 2 different types of fermentation; both of them fermented dextrose and lactose, but only one fermented mannitol.

Lipinski, W. (1923), on the other hand, could not demonstrate any fermentation at all.

Unfortunately, these works are only available in summaries, in which nothing appears about the numbers and ages of strains, media, etc.

The following original works have been found:

Hababou-Sala, J. (1925) demonstrated fermentation of glucose, saccharose and maltose. Three strains were examined, one of which also showed fermentation of lactose and mannitol, after passage through mice.

Assis, A. de (1926) found fermentation of glucose in a 10 % fluid serum medium. Excellent growth in the medium resulted, and when 0.5 % glucose was added, acidification occurred at such a rate that the proteins precipitated.

As to the other biochemical reactions, only the following works have been found: —

Hababou-Sala in the abovementioned work found no coagulation of milk, whereas Nicolau, S. and A. Banciu (1926) could demonstrate growth in ordinary whole milk with neutral reaction, and acidification during growth (pH not stated) which caused the milk to coagulate.

*Author's Investigations.**Strains:*

14 strains were examined — the same as those examined in the above-mentioned work on the sensitivity to chemotherapeutics. After isolation, the strains were dried ad modum Flosdorff & Mudd and kept in vacuum ampoules at $+4^{\circ}$ C. For isolation and drying, reference is made to the author's previous works (1) and (2).

Media:

All the belowmentioned reactions were performed by cultivation in one of the following 3 media or modifications thereof: —

(1) Solid medium consisting of nutrient agar with 1 % peptone and 30 % defibrinated sheep blood. Cultivation was made on agar plates in Petri dishes on which growth will only occur when incubation is made in an atmosphere of saturated water vapours. This was attained by placing the Petri dishes in closed glass jars with a surplus of water on the bottom.

(2) Semifluid medium consisting of thin agar with 30 % defibrinated sheep blood. This medium must be of a consistency that will cause the blood, on standing, to sediment enough to form a clear fluid layer of about $\frac{1}{2}$ cm at the top of the medium. The growth occurs where this layer meets the blood. With the Japanese agar now in use, the composition is: 70 ml 2.5 % agar + 300 ml defibrinated sheep blood + 700 ml broth.

(3) Furthermore, broth with 20 % defibrinated sheep blood was used. This medium was inoculated after sedimentation of the erythrocytes, and growth took place on top of the layer of erythrocytes.

Incubation was made at $33-34^{\circ}$ C.

Maximum growth was found after 3 days' incubation on solid medium, and after 4–5 days in fluid medium.

The following reactions were examined:

Fermentation of carbohydrates. As these reactions are of special interest to the classification of types and differential diagnosis, a number of carbohydrates has been examined:

The pentoses: arabinose and xylose; *the methyl pentose:* rhamnose; *the monosaccharides:* galactose, glucose, and mannose; *the disaccharides:* lactose, maltose, melibiose, saccharose, and trehalose; *the trisaccharides:* melezitose and raffinose; *the polysaccharides:* dextrin, glycogen, inulin, and starch; *the glucosides:* arbutin, salicin, esculin; and finally *the alcohols:* adonite, dulcitol, erythritol, glycerine, inositol, mannitol, and sorbitol.

The mentioned substances were used in 0.5 % concentrations, and phenol red was used as indicator; but this is explained in greater detail in the following.

Fermentation on solid medium.

The first examinations were made with cultivation on a solid medium consisting of nutrient agar with 1 % peptone and 30 % sheep serum. In comparison with 30 % blood agar plates, this medium gives

poor growth of the Ducrey bacilli, but it is necessary to replace the blood by serum in order to ascertain any changes in the colour of the indicator. Ample inoculation was used, and the tests were read after 4 and 6 days' incubation.

Result: None of the strains will ferment any of the mentioned carbohydrates on solid serum medium.

Fermentation in fluid medium.

On account of the varying reports on fermentation of carbohydrates in fluid media, considerable work was performed in an effort to clarify these problems.

The usual semifluid sheep blood agar will ferment a variety of sugars spontaneously, and a number of experiments was therefore carried out in an attempt at finding a suitable semifluid medium. These examinations show that when blood is heated for a couple of minutes on a 100° C water bath until it becomes brown, it will lose its capacity to split the carbohydrates, but at the same time a thermolabile growth factor is destroyed and the growth consequently reduced considerably. As far as is known, the existence of this thermolabile growth factor has not hitherto been demonstrated. On a solid medium with 30 % heat-treated sheep blood a reduction is observed in the size of the colonies to about one third of normal, and the colonies assume the same wrinkled appearance and blurred, indented outline as that observed in cultivations on serum plates. It is also found that whereas all the strains will grow on plates with heat-treated blood, they will grow rather haphazardly in semifluid media with heat-treated blood (the percentage of blood was reduced to 10 on account of the consistency) showing growth and no growth at random, in spite of ample inoculation. On a corresponding medium without agar, only consisting of ordinary broth with 10 or 20 % heat-treated blood, no growth or only microscopic growth is observed.

Already in 1937 *Lwoff, A.* and *I. Pirotsky* demonstrated that the V factor is of no importance to the growth of Ducrey's bacillus, and *Beeson, P. B.* arrived at the same result in 1946. A verification of these experiments has been attempted by producing the V factor by water extraction of ordinary baker's yeast ad modum *André & M. Lwoff* (1937). The extract was filtrated through a Jena glass filter, and its effect examined by means of the filter paper disc method. The medium was plates with 30 % heat-treated sheep blood. No effect of this extract was observed in the experiments. Similarly, it was observed that neither urine, gall or ascites fluid contain the thermolabile growth factor required for the Ducrey bacillus. These experiments must presumably have established that the V factor is not identical with the thermolabile *Hemophilus Ducreyi* growth factor.

Thus it is not possible to produce a medium which will give full

growth of the Ducrey bacilli without using fresh blood, but as the latter will split a number of carbohydrates spontaneously — xylose, rhamnose, glucose, mannose, maltose, saccharose, melezitose, raffinose, dextrin, glycogen, starch, salicin, esculin, and sorbitol — it will not be possible to obtain information about any fermentation of these substances by using such a medium. To avoid spontaneous fermentation of carbohydrates, it is necessary to use heat-treated blood, but as the growth is so very much better in fresh blood, the fermentation reactions were nevertheless examined by cultivation in semifluid agar with 30 % non-heat-treated sheep blood, carbohydrates and phenol red. For these experiments 2 different kinds of control tubes were used, partly tubes with media as mentioned, and partly tubes with the same media and indicator, but without carbohydrates. The former control tubes were not inoculated, whereas the latter were inoculated just like the actual fermentation tubes. In these experiments it was found that the tubes with the carbohydrates just mentioned showed the same changes in colour as the controls not inoculated, and also that these colour changes reached their maximum before the cultures were fully grown. In the other fermentation tubes there were no certain colour changes, although sporadic traces of a yellow colouration were observed. As, however, changes also occurred — likewise sporadically — in the inoculated controls without carbohydrates, the reactions must be considered negative, which means that arabinose, galactose, lactose, melibiose, trehalose, inulin, arbutin, adonite, dulcitol, erythritol, glycerine, inositol, and mannitol are not fermented by cultivation in fluid medium of optimal composition.

In order to examine the fermentation reactions also of carbohydrates fermented by the fresh blood, cultivation was made in semifluid agar with 10 % heat-treated sheep blood to which was added all of the above-mentioned 27 carbohydrates. The tubes were amply inoculated with 3—4 colonies per tube. Only poor growth was observed in these experiments, and the colour changes observed were so faint and uncertain after 4 and 7 days' incubation that all the fermentations must be considered negative.

Although the examinations of the thermolabile growth factor clearly indicate that no appreciable growth may be expected in media without fresh blood, the fermentation of glucose in a variety of media was examined in order to leave no possibility untried. As was to be expected, it appeared that there was no colour change in 100 % sheep serum with an addition of 0.5 % glucose and phenol red. Cultivation in a blood extract produced ad modum Wollstein — *Holm* (1948) — with additions of from 0—90 % broth, as well as cultivation in broth with heat-treated blood as described above, showed minimal or no growth, and no fermentation was observed in these media.

As stated, the examined carbohydrates were added to the media in the concentration of 0.5 %. For glucose it was examined whether

an increase of the concentration would produce better results, but the experiments showed that already 1 % will cause some inhibition of growth, which, however, only becomes really pronounced at concentrations of 2 % or more. The fermentation results are unchanged by this variation of the glucose concentration.

As indicator was used phenol red in the concentration of 12 ml of the dilution 1/500 per litre of medium. Experiments with indicators causing colour changes at lower pH-values (bromothymol blue, bromocresol purple, and Andrade's indicator) also show unchanged results.

Attempts at titrating the amounts of acid formed in the tubes in order, if possible, to show a difference in the formation of acid between the control and the fermentation tubes indicate that this cannot be done with any degree of accuracy on account of the consistency of the semifluid medium. As mentioned, growth takes place where the uppermost clear layer meets the sedimented blood; but after 5—6 days' incubation, the consistency of this layer is like that of thick jelly. In this connection it should also be noted that the colour change in the control tubes, as already pointed out, quite obviously takes place without any relation to the growth.

Result: None of the strains will ferment carbohydrates in the used fluid media.

Voges-Proskauer's test.

Medium: Partly the usual semifluid medium with 30 % sheep blood, and partly the semifluid medium mentioned under the fermentation reactions, with heat-treated blood to which was added 0.5 % glucose.

After 6 and 7 days' incubation 1 ml of 10 % KOH was added per tube, and the test was read 18—20 hours later (positive reaction indicated by pink fluorescence). It should be noted that the growth in the latter medium is very poor.

Result: None of the strains show positive Voges-Proskauer test.

Coagulation of milk.

As stated in the introduction, Nicolau & Banciu have had growth and coagulation in milk. As in spite of repeated tests, the author did not succeed in making his strains grow in whole milk or skimmed milk (sterilised ad modum Koch) he tried cultivation partly in whole milk and skimmed milk with additions of 20 % defibrinated sheep blood, and partly in semifluid agar with 30 % sheep blood to which, after sedimentation of the blood, was added sufficient milk or skimmed milk to dilute the uppermost clear layer by about 50 %. In spite of ample inoculation — 4—5 colonies per tube — growth occurred only in the latter medium. After 6 days' incubation there was not the slightest

coagulation in the milk. The tests were repeated several times, and in one case incubation was made for 10 days, without any change in the result. With regard to the abovementioned thermolabile growth factor cultivation was also tried in Berkefeld-filtrated non-heat-treated milk, but the Ducrey bacilli would not grow in that medium either.

Result: None of the strains will grow in or will coagulate milk.

Oxidase reaction.

Reagent: Tetrametyl-p-phenylenediaminhydrochloride 1/200.

The reaction was performed both with plate culture (incubated for 3 and 4 days) and cultures in semi-fluid sheep blood agar (incubated for 4 days). The result was read at once and 5—10 minutes after addition of the reagent.

Result: Negative reactions for all strains.

Indole formation.

Indole reagent: paradimethylamidobenzaldehyd	8 g
96 % alcohol	760 ml
concentrated H Cl	160 ml

Cultivation in semifluid sheep blood indole agar consisting of indole broth (1 part of trypsin-digested casein broth + 2 parts of ordinary broth) + 30 % defibrinated sheep blood + agar.

When the culture was 4 and 6 days old the reagent was added in such a way as to make it settle on the surface of the medium. Positive reaction is indicated by the formation of a red ring where the 2 layers meet.

All the strains showed poor growth in the medium. A repetition of the test with still more inoculation of the media gave no change in the result.

Result: None of the strains form indole.

Formation of hydrogen sulphide.

Medium: Semifluid 30 % sheep blood agar with addition of 0.1 % lead acetate. Formation of hydrogen sulphide will be seen by black colouration of the medium. As there was excellent growth in this medium, no other methods were tried.

Result: None of the strains form hydrogen sulphide.

Nitrate reduction.

Medium: Semifluid sheep blood agar with addition of 0.1 % potassium nitrate. 0.5 ml alfanaphthylamine + 0.5 ml sulfanilic acid was

added to the culture after 4 and 6 days' incubation. Positive reaction is seen by purple colouration, whereas uncoloured medium means negative reaction.

Result: None of the strains will reduce nitrate.

Protein liquefaction.

Medium: Semifluid 30 % sheep blood agar with addition of slices of heat-coagulated white of hens' eggs.

Positive reaction is indicated by varying degrees of solution of the slices.

Result: None of the strains will liquefy the white of hens' eggs.

Liquefaction of sheep serum.

The procedure is exactly like the one adopted in the above test.

Result: None of the strains will liquefy sheep serum.

Methylene blue reduction.

Medium: Semifluid 30 % sheep blood agar.

After 4 and 6 days' incubation, 1 drop of watery 1 % methylene blue solution was added to the cultures; the test was read after 1, 4, and 24 hours.

Result: None of the strains will decolourise methylene blue.

Gelatine melting.

Medium: 30 % defibrinated sheep blood in gelatine. The gelatine was composed as follows: Bullox meat extract $\frac{3}{4}$ %, Parke Davis peptone $2\frac{1}{2}$ %, NaCl $\frac{1}{2}$ %, and gelatine 10 %. The medium was cleared by addition of whipped white of eggs, and pH adjusted at 7.6.

The medium was inoculated while the gelatine was heated to 33° C and after sedimentation of the erythrocytes. There was excellent growth on top of the layer of erythrocytes. After 4, 6 and 8 days' incubation the tubes were placed in an icebox in order to see if the cultures had melted the gelatine. That solidifies the gelatine completely.

Result: None of the strains will melt gelatine.

Formation of ammonia.

Medium: Semifluid 30 % sheep blood agar.

Nessler's reagent was added after 5 and 7 days' incubation, which will cause the medium to become slightly brown, but not more than the control tubes.

Result: None of the strains will form ammonia.

Discussion.

The tests clearly demonstrate that according to the usual methods of examination used in practical bacteriology *Hemophilus Ducreyi* shows little biochemical activity. But that is also of great importance, inasmuch as negative reactions are just as valuable as positive ones for distinguishing apparently closely related bacteria.

After the mentioned experiments on fermentation reactions in solid and fluid media, it seems surprising that Assis could demonstrate fermentation of glucose for his strains. Unfortunately, his work does not mention anything about the number, possible differences in the fermentation, or the age of his strains. Assis's medium was heated to 110° C for 15 minutes, and it is therefore impossible that it can have had any traces at all of the thermolabile growth factor; in view of the excellent growth in his experiments it is probable that he worked with old, dissociated laboratory strains. In his work Assis also points out that the tested strains were Gram-positive in alcohol-fixed preparations, but Gram-negative in films fixed by heating. All the strains tested in this work are Gram-negative in alcohol-fixed preparations. This relation to the Gram-staining might give rise to a suspicion that Assis's strains may not have been *Ducrey bacilli* at all, but later works by the same author show that there can hardly be any doubt as to the identity of the tested strains.

Kamimura's and Lipinski's results cannot be evaluated in detail, because the works are only available in summaries with very little information about media, etc. For his strains Hababou-Sala states that they are strictly aerobic and give excellent growth in Besredka's medium. As for the author's own strains, they are facultatively anaerobic and give only microscopic growth in Besredka medium, in spite of very ample inoculation. The explanation of the differences in the results of the fermentation reactions must presumably be that some of the strains dealt with in literature are dissociated laboratory strains.

It is not claimed that the fermentation of carbohydrates by the strains has been solved by the reported investigations. But no further progress is likely to be made until more is known about the thermolabile growth factor, and particularly until it has become possible to separate it from the carbohydrate-fermenting enzymes which are also found in the blood. Assis's contention to the effect that the strains really ferment glucose can thus not be refuted until it becomes possible to cultivate on a medium which contains the growth factor.

With the fermentation reactions it has only been tested whether the strains will ferment the carbohydrates under formation of acids, so that the indicator will register colour changes, whereas the tests do not state whether the substances are digested in other ways. An investigation of this problem would be of great interest, also for the classification of types, but unfortunately such an investigation cannot

be carried out by the author — nor can he go into a detailed investigation of the thermolabile growth factor.

None of the biochemical reactions contribute to the solution of the question of types: details about these problems will be given at a later date.

Nicolau & Banciu's strains, which provoke coagulation when growing in milk, are probably also dissociated laboratory strains. The authors were among the first to demonstrate that repeated reinoculations will cause *Hemophilus Duereyi* to dissociate, and that strains of a certain age will grow on media where freshly isolated strains will give no growth whatever. Unfortunately, it does not appear from their works whether it was such laboratory strains that were used for the reported investigations.

Summary.

The biochemical reactions of 14 *Hemophilus Duereyi* strains were examined. The results of the tests are that all the reactions generally used are negative. In regard to fermentation of carbohydrates nothing can be said with any certainty until it is possible to isolate a thermolabile growth factor, about which some details are given.

In regard to growth in milk, fermentation of glucose, etc. conformity is not found with the results previously reported in literature. It is stated that these investigations were undoubtedly carried out with old, dissociated laboratory strains.

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IRRADIATION OF FOOT AND MOUTH DISEASE VIRUS WITH ULTRA-VIOLET RAYS

By *S. Schmidt, A. Hansen & P. Holm.*

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The past few years have witnessed the publication of several communications reporting that some viruses, on being irradiated with ultra-violet light, are transformed into non-toxic modifications capable of producing immunity when injected into the animal organism.¹⁾ Among these viruses are those of rabies and psittacosis. By means of the irradiation method it has been possible to prepare vaccines against rabies which, in animal experiments (mice) seem to be more active than those hitherto used in practice, i. e. virus attenuated by desiccation or inactivated by chemical compounds, especially phenol.

As far as we are aware, no experiments have been performed with foot and mouth disease virus, for which reason we considered we ought to examine whether this virus could be inactivated by this means, whether the inactivated substance had immunizing properties, and whether these properties were just as good or perhaps better than those possessed by the vaccine now employed (Schmidt-Waldmann). In the following we shall quite briefly report on the results of our studies of the pure fundamentals; a comparison of the effects of merely two vaccines being rather circumstantial, time-consuming and very exacting as regards the number of experimental animals (guinea-pigs and cattle), because as yet there is no true standardization method for vaccine against FM.²⁾

¹⁾ We shall refrain from any complete historical retrospect and mention merely a few of the most recent works which have been of particular interest in our studies: H. Habel & B. T. Sockrider, *Journ. Immunolog.* 1947, 56, 273; K. Habel, *Publ. Health Rep.* 1947, 62, 791 and R. D. Francis, Al Milzer & F. B. Gordon, *Proc. Soc. Exp. Biol. & Med.* 1947, 66, 181. Franz Oppenheimer and S. O. Levinson: A new method for the production of potent inactivated vaccines with ultraviolet irradiation. 1. Principles, technic and apparatus. Publication withheld by the Committee on Medical Research of the Office of Scientific Research and Development.

²⁾ FM = foot and mouth disease.

Apparatus and Technique.

Placed in the centre of a triangular metal stand of stainless steel is a silica tube with a diameter of 5 mm. and a length of 90 cm.

At each corner are three cylindrical lamps, so that they are equidistant from one another (120°). The distance from the outer wall of the lamps to the silica tube is 15 mm. The lamps are Westinghouse sterilamps WL and are 80 cm. long. For the protection of the experimenters the entire apparatus is enclosed in a steel jacket which is easily removed when work is finished. In the upper end of the silica tube is a separating funnel in which is mounted an electrically driven agitator. The lower part of the tube ends in a fine glass nozzle fitted on with a length of rubber tubing which is fitted with a stop-cock.

In order to determine the irradiation time the separating funnel is filled with water, the cocks are opened so that the silica tube is filled up, whereafter the stop-cock in the bottom is closed. The lamps are switched on, a stop-watch is started and the stop-cock opened, a flask having been placed under the glass nozzle just before. The time taken for 100 c. c. to run through the tube when the cock is wide open is then determined. The irradiation time can be varied by regulating the stop-cock.

For the first tests our initial material was a virus suspension (virulent guinea-pig or cattle lymph) containing 1.4 % virus adsorbed to aluminium hydroxide.¹⁾ The quantity of the latter was 50 %, corresponding to 0.9 % Al_2O_3 . This adsorbate is of the same composition as that forming the initial point for vaccine production by the Schmidt-Waldmann method, in which is also added formaldehyde, whereafter the mixture is heated to 26° for 60 hours, then homogenized, etc.²⁾

Prior to irradiation the adsorbate is diluted with 9 parts of freshly distilled, carbonic acid-free water; the agitator in the separating funnel is started and irradiation proceeds as already described, the time being varied. 100 c. c. diluted adsorbate was used in each irradiation test.

1. Experiments with Guinea-Pig Virus.

The virus used was of type A Vallée, and the titre, i. e. the smallest quantity of virus capable of inducing FM when injected intracutaneously into guinea-pigs, was 10-7.

The results of some tentative experiments showed that the virus lost its infectivity when irradiated for a certain time, but retained a greater or smaller power of immunization according to the duration of the irradiation.

When the irradiation time is too short there are still toxic remnants in the adsorbate; if it is too long the immunizing power is destroyed.

¹⁾ The same preparation employed in preparing vaccine for practical use.

²⁾ S. Schmidt: *Maanedsskrift f. Dyr læger*, 1941, 53, 1.

S. Schmidt & A. Hansen: *Maanedsskrift for Dyr læger*, 1944, 56, 73.
By selecting the correct irradiation time one obtains non-toxic ad-

sorbates of a high immunizing effect, i. e. the virus has been transformed into vaccine.

We shall reproduce the results of a single experiment, tabularized below:

Table 1.

Exposure in seconds	Test of					
	infectiveness			immunity		
540	0	0	0	—	—	—
	0	0	0	—	—	—
360	0	0	0	—	—	—
	0	0	0	—	—	—
195	0	0	0	—	—	—
	0	0	0	—	—	—
75	0	0	0	—	—	—
	0	0	0	—	—	—
65	0	0	+	—	—	
	+	+	+			
45	+	+	+			
	+	+	+			
21	+	+	+			
	+	+	+			
10	+	+	+			
	+	+	+			

+ = FM

— = immunity.

The first column of the table shows the irradiation time in seconds. The next two contain the results of the tests of infectiveness and immunity respectively. Six guinea-pigs were employed in each test. Each animal was injected with $\frac{1}{2}$ c. c. intramuscularly in each hind leg and three intracutaneous injections in each planta, a very severe test. It will be seen that the samples exposed for 540, 360, 195 and 75 seconds were all harmless, the symbol O signifying that neither local (i. e. on plantae) nor general signs of FM were observed.

The sample which had been exposed to irradiation for 65 seconds still contained a small residue of virus, as two-thirds of the animals acquired FM, the other third manifesting no symptoms.

The remainder of the samples, which had had a still shorter exposure, viz. 45, 21 and 10 seconds, were all highly toxic, all the injected animals acquiring typical, generalized FM.

The animals that had no FM symptoms after injection with the irradiated adsorbate were subjected to an immunity test eight days later, 10,000 infectious virus doses being injected intracutaneously into one planta. Six fresh animals received the same dose. The latter developed typical, generalized FM in the course of the next few days, whereas the vaccinated animals had merely local reactions of varying intensity at the injection site, but in no case manifested generalized disease (secondary aphthae, loss of weight, salivation, loss of appetite, etc. which characterize the disease).

2. Experiments with Cattle Virus.

We need describe only a single experiment, comprising a very small number of animals.

We employed lymph prepared on 3 cows by intralingual injection and collecting the material 24 hours later. The titre for cattle was 10^{-7} , for guinea-pigs 10^{-3} , i. e. moderately virulent for the latter animal. An adsorbate containing 1.4 % virus and 50 % $\text{Al}(\text{OH})_3$ was prepared in the usual manner, then diluted with 9 parts of water and irradiated for varying times. The different samples were then injected into guinea-pigs by the usual method.

Using the two samples which in the guinea-pig test had given the clearest results, i. e. those exposed for 165 and 60 seconds respectively, we injected 6 cows, three with each sample. All the animals were injected intralingually (the most sensitive site) and *also* subcutaneously with 100, 60 and 30 c. c. After fourteen days they were again injected (except the one that developed FM, see table) intracutaneously in the tongue with 10,000 times the virus dose capable of producing generalized FM in non-immunized animals. The experiment¹⁾ gave the following result:

Table II.

	Injection dose	No. of cows	Tests of	
			infectivity	immunity
A. Sample irradiated 165 seconds	1 c. c. intra-lingual + 100 c. c. subcut.	1	0	moderate local reaction at inj. site, but no gen. symptoms of FM
	1 c. c. intra-lingual + 60 c. c. subcut.	1	0	total immunity
	1 c. c. intra-lingual + 30 c. c. subcut.	1	moderate gen. FM	not tested
	untreated cow (control)	1		gen. FM.
B. Sample irradiated 60 seconds	1 c. c. intra-lingual + 100 c. c. subcut.		0	total immunity
	1 c. c. intra-lingual + 60 c. c. subcut.		small primary aphtha at inj. site, but no gen. symptoms	total immunity
	1 c. c. intra-lingual + 30 c. c. subcut.		0	total immunity
	untreated cow (control)	1		gen. FM

¹⁾ The pH of the virus suspension before irradiation was 6.5 and was *not* regulated later. Thus the vaccine had a slightly acid reaction.

It will be seen that there is fairly close agreement between these results and those obtained with guinea-pigs. Detoxication was not complete; but, having regard to the severe test to which the animals were exposed: injections into the tongue and into the subcutaneous tissue there can only have been small toxic residues in the irradiated adsorbates. On the other hand, the immunity here was more pronounced than in the guinea-pig tests, as in four cases there was not even a local reaction at the injection site; this agrees with the fact that cattle virus is a better antigen to cattle than to guinea-pigs. It was remarkable that an animal which had received only 30 c. c. (= 3 c. c. undiluted vaccine) was also immune. In fact, in another experiment we were able to immunize a cow with 3 c. c. (corresponding to 0.3 c. c. undiluted vaccine). This shows that irradiated adsorbate possesses a particularly high immunizing effect.

Irradiation experiments with virus diluted in saline containing m/500 phosphate buffer at pH 7.6 showed that the virus was transformed in the same manner to anavirus. This too has a powerful immunizing action, but the immunity is of relatively short duration. If adsorbates are prepared from these anaviruses we get vaccines with a more powerful action (i. e. they immunize in smaller doses) and giving longer immunity than the irradiated virus alone.

Conclusions.

The virus of foot and mouth disease, when irradiated with ultraviolet light under suitable experimental conditions, is transformed into a non-toxic modification (anavirus) which possesses distinct immunizing properties. This applies to both guinea-pig passage virus and cattle virus, and the virus behaves in practically the same manner whether we use a simple dilution in saline or an adsorbate. The immunizing properties of a detoxicated virus dilution can be intensified by adsorption to aluminium hydroxide.

Investigations are in progress regarding a comparison of the action of these vaccines with the action of formalized and heat-treated adsorbates, as well as experiments on perfecting the technique of irradiation.

POST-MORTEM FINDINGS IN PENICILLIN-TREATED SUBACUTE BACTERIAL ENDOCARDITIS

(Preliminary Report of 6 Unselected Cases).

By *Erkki Saxén*.

(Received for publication December 2nd, 1948.)

Subacute bacterial endocarditis used to run a fatal course within a few months, although it could last a year and even longer. Yet after the sulpha preparations came into use, frequent recoveries were published and it appears now that several cases of subacute bacterial endocarditis can be made to recover on a penicillin medication, provided the treatment is initiated in time and adequate dosage is applied for a sufficient duration. In recent times streptomycin has also been used with encouraging results (*Guss* 1948).

The comparatively short time of observation tends however to render an assessment of the results difficult so far. It must be taken into account that even if the bacteria disappear from the blood while the treatment is in progress, they may possibly continue alive in vegetations which are difficult of access to the bactericide, owing to the surrounding layers of fibrin. Frequent relapses argue for this, to which those patients are especially liable who have been treated with small amounts of penicillin. The results of treatment were also claimed to have been impaired by the circumstance, that the scar formations developing during the treatment lead to severe heart insufficiency which subsequently causes death. (*Bloomfield et al.* 1945).

An attempt is made to throw some light on these circumstances by publishing below six unselected cases of subacute bacterial endocarditis with pronounced clinical features, treated with penicillin and examined post-mortally.

The treatment was incomplete and unsystematic, (the patients having been treated at different hospitals), since at the time there was but little experience of a penicillin medication and a severe shortage of the drug. In dealing with the cases, attention is mainly directed to the patho-anatomical changes found in the heart and to

such findings in other organs which may be taken to illustrate the activity of the disease. In addition, bacterial stainings have been carried out in histological preparations in order to detect bacterial colonies.

Case 1. A male, aged 27, who as a child developed heart disease in consequence of angina, was hospitalised with a history of fever for about a year. He had received at first some sulphur and penicillin cures, which always abated the fever for some time. The blood cultures were negative but the diagnosis of endocarditis lenta was supported by a prolonged febrile course, mitral lesion, patent ductus arteriosus, skin embolies, hematuria, high sedimentation rate (SR 140) and secondary anemia (Hb 40). Finally the patient was administered penicillin 250,000 to 1 million units per 24 hours for a time of 11 weeks, in all 20 million units. The fever fell at once after the initiation of the treatment and the general condition improved but the sedimentation rate continued high (120) and the Hemoglobin level could not be made to rise. After the ligation of the ductus arteriosus the patient died suddenly, having been afebrile for a period of 16 weeks.

Post-mortem examination revealed a dilated heart, weight 390 g. The mitral valves were thickened and showed several wartlike growths the size of a pin's head, from which small parts were easily detached. The valves were thin and the chordae tendinae normal. The aortic valve revealed no changes. Distally to the arteria subclavia sinistra the ductus began from the aorta 3 mm long and 4 mm wide. It was firmly ligatured and no macroscopic inflammatory signs were found in the vicinity. The spleen was enlarged, weighed 380 g. and exhibited an infarct the size of a finger tip. The kidneys had a slightly granular surface with hemorrhages. The cortical layer was reduced and of a diffuse structure. Both lungs were atelectatic and the left pleural cavity filled with about half a litre of dark bloody exudate. Chronic tonsillitis was found in addition.

The *histological* examination gave indications of chronic focal nephritis, a recent infarct in the spleen and chronic tonsillitis. The pieces taken from the heart revealed a certain extent of fibrosis in the myocardium, particularly round the blood vessels, which were engorged in places. No signs indicative of recent inflammation were disclosed in the heart muscle, and no Aschoff's bodies. The mitral valve was thickened and fibrotic, revealing a significant amount of small nodes formed from granulation tissue, covered in places by a thrombus mass in the process of organisation. Here and there a thin fibrin layer was seen direct on the surface of the connective tissue. This latter was firm, hyalinised, with very few nuclei. There were no cellular reactions in the mitral valves. Specimens taken from ductus walls revealed that they were covered by a thrombus containing a large quantity of granulocytes. The margin of the thrombus and the wall had abundant granulation tissue and polymorphonuclear leukocytes. Staining by Gram's method did not reveal bacteria either in the heart muscle or in the valves, but colonies of bacteria were seen in the walls of the ductus. These bacteria were Gram-positive cocci, present in relatively small quantities and not staining readily, nor could any visible grouping be found. Bacteria were not seen in any samples taken from other organs.

Case 2. A male, aged 37. At the age of 22 he was found to have dilatation of the heart and gave now a history of sweating, weakness and slight feverishness for over a year's time. The diagnosis Endocarditis lenta was made on the basis of a prolonged febrile state, aortic lesion, hematuria, anemia (Hb 45) and enlarged spleen. The blood cultures were negative. The patient was administered penicillin for a period of 5 weeks, 800,000—1,000,000 units 6 times in 24 hours, totalling 20 million units, and after that streptomycin 0.25 g.

6 times in 24 hours, 9 g. in all. The fever abated at once on the second day after the penicillin medication was started and there was no relapse for 9 weeks, when death occurred. While the penicillin treatment was in progress, the patient's heart insufficiency symptoms were continually aggravated and the diastolic murmur was intensified. The patient succumbed to heart failure. Sedimentation rate persisted high for the whole time of the treatment (70 mm/h.)

The *post-mortem* findings lent support to the endocarditis lenta diagnosis. The heart was enlarged, weighed 700 g., the heart muscle was firm, thickened throughout and the section revealed a greyish-brown surface. The cavities were strongly dilated and contained blood coagula. The inner membrane was thin. The anterior cusp of the mitral valve contained two round cysts the size of a pea, with a smooth surface and filled with blood. The aortic valve showed low corrosions and in the thickened and partly scarred free margin numerous excrescences resembling cauliflower, with a mainly firm but in places somewhat softer consistency. Thrombus mass was also found here and there. Stasis was found in all the organs. The liver weighed 2400 g., the spleen 400 g. and it had a couple of small infarcts. Serous fluid was abundant in the pericardium and the pleural cavities. The kidney exhibited signs indicative of focal nephritis, and there was edema in the brain.

Histological examination of specimens taken from the heart revealed that the cysts of the anterior cusp of the mitral valve were the size of a pea containing blood but no signs of an active inflammation. The aortic valve was found to be strongly thickened. It consisted of a firm partly hyalinised connective tissue and its surface revealed in places elevations formed by granulation tissue, and in places scarred depressions. Endothelium could not be distinguished but immediately covering the connective tissue there was a layer of fibrin of variable thickness. No inflammatory cells were found in the aortic valve.

Gram-staining revealed on the boundary between connective tissue and fibrin layer bacterial foci, formed by Gram-positive cocci. There was no visible grouping, (fig. 1).

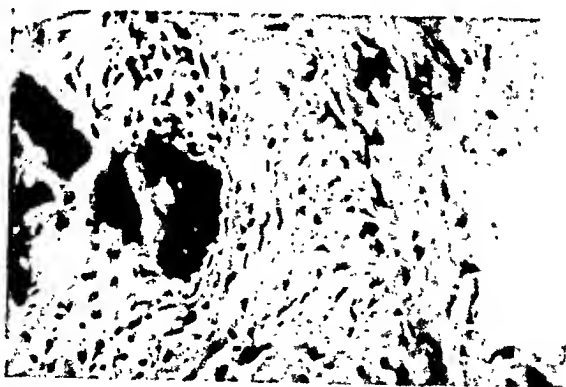


Fig. 1.

Case 2. Aortic valve showing scar tissue, bacterial colony but no active inflammation (van Gieson stain).

Case 3. A female, aged 20. As a child she had had scarlatina which left her with a valvular disease. Half a year before hospitalisation she had been ill with «influenza» and run a temperature since then. *Streptococcus viridans* grew repeatedly in the blood cultures, and a diagnosis of endocarditis lenta

was further supported by persistent fever, mitral lesion, enlarged spleen, Osler's nodes and anemia (Hb 48). The patient was administered penicillin 30000 units 8 times daily, altogether 3.8 million units. The fever subsided on the second day, but rose again in a fortnight, when the patient died with symptoms of cerebral embolism. Sedimentation rate persisted at 70 to 80 for the whole time of hospitalisation.

The *post-mortem* findings supported the clinical diagnosis. The heart was considerably enlarged in every direction and weighed 400 g. Both ventricles, the left especially, were strongly dilated and the walls somewhat hypertrophied. The left atrioventricular opening was 4 fingers wide. The free margin of the bicuspidal valves was torn and covered with thrombus masses, whereas the other valves did not reveal any changes. The spleen was enlarged, weighed 600 g. and its pulp was disintegrating. In addition stasis was found in liver and lungs, the lungs revealed edema, the kidneys signs typical of nephritis and there was chronic tonsillitis. The left parietal lobe of the brain revealed an emolition focus the size of a woman's fist, containing a pulpy mass. Histological specimens were not taken in this case.

Case 4. A male, aged 31, who for a time of two years had suffered from lassitude, 2 months prior to hospitalisation he came down with diarrhea and fever, which latter had persisted since. Blood cultures were negative, yet a diagnosis of endocarditis lenta was established on the basis of prolonged fever, aortic and mitral lesion, hematuria, Osler's nodes and anemia (Hb 55). The patient was administered penicillin for a time of 5 weeks 30000 units 6 times per 24 hours, altogether 8 million units. During penicillin treatment the sedimentation rate decreased from 80 to 13, but the hemoglobin level remained low. Dyspnea was continually aggravated, systolic and diastolic murmurs were intensified and the stasis rates increased. Having been afebrile for a time of 8 weeks, the patient died with symptoms of heart insufficiency and pulmonary edema.

The *post-mortem* findings supported the clinical diagnosis. The heart was enlarged, weighed 570 g. The atrioventricular openings were wide and the cavities dilated. The aortic valve was covered with a thick thrombus mass and was thickened but whole. Additional findings: ascites, an enlarged liver with stasis, pulmonary edema, nephritis and furthermore infarcts in liver, kidneys and spleen.

The *histological* examination brought to light that the muscle cells of the myocardium were somewhat hypertrophied and there was slight fibrosis, but no signs indicating recent inflammatory processes. The aortic valve showed abundant verrucose nodes formed by loose or partly hyalinised connective tissue and firmer scar formations. The endothelium was also thickened in places. No signs of active inflammation were found. The kidneys revealed an old infarct and signs of chronic focal nephritis.

Gram-staining demonstrated in places bacterial colonies in the connective tissue of the aortic valve under the endothelium, but no cellular reaction in their vicinity. The bacteria stained strongly in places and were revealed to be Gram-positive cocci, whereas they partly stained very poorly. (fig. 2).

Case 5. A male, aged 38, who as a child had had angina after which a heart lesion was ascertained. He had contracted influenza 2 months prior to admission and been feverish since then. The sedimentation rate had varied between 80 and 110. At the hospital the patient was found to be suffering from anemia (Hb 55), a patent ductus arteriosus, aortic and mitral lesion, hematuria and enlarged spleen; on the basis of these findings a diagnosis of endocarditis lenta was established, although all blood cultures were negative. The patient was administered penicillin 30000 units 8 times per 24 hours over a

period of 5 weeks, or in all 8 million units, and the fever fell already on the second day. When the patient had continued afebrile for 6 weeks and the sedimentation rate had been brought down to 33, he was discharged. At home he was free from fever for only 2 weeks and when he was readmitted to the hospital having first been feverish at home for 3 months, he was already in a very poor condition. He had septic fever, SR 102, Hb 45, a much stronger

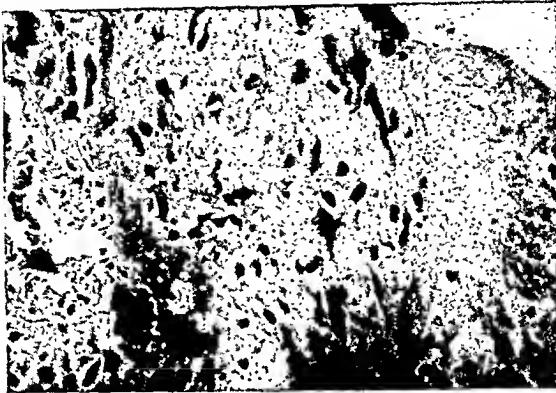


Fig. 2.

Case 4. Aortic valve covered with a thrombus in process of organisation. Bacterial colony, which Gram-staining revealed as Gram-positive cocci. No cellular reactions distinguishable around the colony (van Gieson stain).

dilatation of the heart than before, pulmonary stasis and exudate in the pleural cavities. The patient died in a few days with symptoms of sepsis and heart failure.

Post-mortem examination revealed that the heart was hypertrophied, enlarged and weighing 490 g. The margins of the left atrioventricular valve showed wartlike growths the size of a match head. No changes were found in the other valves. The ductus was 4 mm long and 3 mm thick. The end next to the aorta had a funnel-shaped groove with wartlike formations. Other pertinent findings: enlarged liver with stasis (3500 g.), pulmonary stasis and infarcts in spleen and kidneys. The spleen was enlarged and weighed 610 g. The kidneys revealed changes characteristic of nephritis, and both lungs pneumonic foci.

The *histological* examination of sections taken from the liver, kidneys and heart supported the post-mortem findings. There was abundant fibrosis in the myocardium and in places, as signs of an active inflammation, accumulations of polymorphnuclear leukocytes. The mitral valve was thickened, revealing locally nodes formed by a firm and partly hyalinised connective tissue and depressions lined by a thin layer of fibrin. In places ulcerations and thrombi were seen, abundantly containing polymorphnuclear cells. The thrombi were recent in places and in places in the process of organisation.

Gram-staining revealed abundant Gram-positive cocci in the thrombi, both in their marginal parts as well as deeper on the border of the connective tissue. Emboli consisting of bacteria were also disclosed in the kidneys.

Case 6. A male, aged 24; he had had angina at the age of 5 and was said to have a weak heart since then. Subsequent upon a tooth extraction he had become feverish and this state persisted up to his admission to hospital 2 months later. At the time of hospitalisation his general condition was bad, Hb 48, SR 80, he was found to be suffering from a slight heart insufficiency, aortic lesion, enlarged spleen and hematuria. Although the blood cultures

were negative, the diagnosis endocarditis lenta was made on the basis of the facts described above. The patient was administered penicillin 250,000 units daily for 6 weeks, altogether 13 million units. The fever abated on the third day of treatment and did not return for a month. At that time the patient developed a large hematoma on the lower part of the thigh and died primarily in consequence of severe anemia and a poor general condition. The heart insufficiency the patient was suffering from when the treatment was initiated, showed signs of improvement since dyspnea became less severe, the liver decreased in size and the stasis rales subsided. The sedimentation rate remained at between 70 and 80 for the whole time of hospitalisation.

The post-mortem findings supported the diagnosis of endocarditis lenta. The heart was enlarged, weighed 600 g. The atrioventricular openings were wide and all cavities large. The cusps of the aortic valve showed closing margins up to 5 mm thickness with a firm consistency and yellowish red surface. They revealed elevations and ulcerations; these changes continued to the posterior surface of the mitral valve and the chordae tendinae, which were thickened, shrunken and showed cauliflower-like formations as well as ulcerations. The closing margins of the mitral valve cusps had some strongly scarred thickenings. The aorta, coronary arteries and heart muscle presented the usual appearance. The liver weighed 2100 g. and disclosed signs of stasis and fatty degeneration. The spleen was enlarged. Signs typical of nephritis and small old infarcts were found in both kidneys, as well as in the spleen. There was a fresh thrombus mass adhering to the wall at the site of ramification of the left femoral artery, and a blood clot the size of 3 fists was found between the muscles of the anterior surface of the thighs.

The histological examination of the valves, liver and kidneys was consistent with the post-mortem findings. The heart muscle appeared normal, although there was some slight fibrosis in places and slightly hypertrophic cells. There were no signs of recent inflammatory processes nor any Aschoff's bodies. A piece taken from the cusp of the mitral valve revealed scar tissue deficient in cells, but no signs of recent inflammation. Nor did the sections demonstrate any fibrin layers or thrombus mass. The aortic valve revealed in the sub-endothelial connective tissue polymorphonuclear leucocytes, very abundant here and there, and in places bone tissue was seen. The endothelium was strongly thickened, and the tissue exhibited deep ulceration covered with fibrin and thrombus mass. Some thrombi were recent, and some in the process of organisation.

Gram-staining did not reveal any bacteria in the mitral valve, but in the aortic valve, immediately under the endothelium and in the thrombi there were abundant colonies of Gram-positive cocci. The cocci were seen in places two-three in a row, but in general they did not show any grouping. The colonies were sharply limited and no cellular reactions were visible in their vicinity.

On studying the clinical aspect of the cases it becomes evident that although all exhibited unmistakable clinical symptoms of subacute bacterial endocarditis, the blood cultures were positive in one instance only. Common to all was a cardiac lesion, and two cases had in addition a patent ductus arteriosus. Prior to the initiation of penicillin therapy the cases had run a febrile course of 10 to 80 weeks. At the start of the treatment the sedimentation rate of the blood exceeded 70 mm/h in all cases and Hemoglobin of the blood was below 50 with one exception, where it was 55. Before the treatment was

begun heart insufficiency was found in 4 patients, in 3 of whom it was leftsided. The patients all received a penicillin medication, and one was in addition administered streptomycin. The total amount of penicillin varied between 3.8 and 45 million units and the proper penicillin cure lasted from 2 to 11 weeks. It was a common feature to all that the temperature subsided on the second or third day of treatment. In five instances there was no relapse before death occurred, 2 to 16 weeks since the initiation of the treatment. In one case the fever did not return for 8 weeks when it rose again and persisted, since there was no treatment, until death occurred in 12 weeks. From the therapeutic point of view case 1 presented striking features, having exhibited a febrile course for somewhat over a year. This patient was initially administered 15000 units 8 times daily, altogether 6 million units, and was subsequently afebrile for 4 months. Having again run a temperature for 2 months, he was given penicillin 1 million units daily for a week, after which he continued afebrile for a fortnight when his temperature rose again. The patient was immediately subjected to a similar cure and there was no fever for 3 weeks. As it rose again, the patient was administered penicillin 250000—1 million units daily for 11 weeks, during which period there was no temperature. The cause of death was a massive atelectasis after the ligation of the ductus arteriosus. In the remaining cases heart failure was the cause of death for three, brain emboly for one and one died of anemia, partly hemorrhagic anemia. In those four cases who exhibited symptoms indicative of heart insufficiency before the initiation of the therapy, the disease was aggravated in two and was the immediate cause of death, whereas in two it showed signs of improvement, although one of them died later with symptoms of heart insufficiency, having first run a febrile course for 12 weeks in the absence of treatment. Those two cases who had no symptoms of heart insufficiency before the treatment, did not exhibit any while the therapy was in progress.

At autopsy all the cases revealed changes typical of subacute bacterial endocarditis. Thus they all had ulceroverrucose changes in the heart valves, signs characteristic of focal nephritis and infarcts in the spleen. The valvular changes involved the mitral valve in three and the aortic valve in three cases. In five cases the post-mortem findings were supported by histological examinations.

It was then disclosed that in three cases no signs arguing for an active inflammation could be detected in the heart valves. They were thickened and formed from scar tissue deficient in cells and partly from hyalinised connective tissue. In one of these cases there was no fibrin layer on the valves, one had a homogeneous fibrin layer immediately above the connective tissue, and one case revealed thrombi in the process of organization in the valves. It is noteworthy that all cases exhibited verrucose valvular changes in which proliferating connective tissue was found. In the first case whose valves had neither

fibrin layer nor thrombi, the wall of the patent ductus arteriosus showed recent and partly organising thrombus with an abundant amount of granulocytes. Of the remaining two histologically examined cases one had bone tissue on the aortic valve as a mark of a previous inflammatory process; marks of recent inflammation were deep ulcerations covered with fibrin, showing on the borderline of the connective tissue abundant polymorphonuclear leucocytes. This case also had old scar formations of the mitral valve. The second case exhibited on the mitral valve both old scar formations and recent thrombi, abundantly containing granulocytes. Staining revealed bacteria, in addition to these two cases with histological findings of marks of recent inflammation, also in all the other histologically examined cases, although one of them did not exhibit any in the valves, but in the walls of the patent ductus arteriosus. Only one case had abundant bacteria in the thrombi, whereas in all others they were seen in sharply limited colonies between connective tissue and thrombus (fig. 1 and 2). The bacteria were in every instance Gram-positive cocci, staining strongly in three and not so well in the remaining two cases. No distinct grouping could be recognised.

We are therefore able to conclude that, aside from the clinical picture of the cases and the post-mortem findings, the staining of bacteria also supports the theory that the disease was subacute bacterial endocarditis, and in all probability Endocarditis lenta due to *Streptococcus viridans*. The circumstance that the blood cultures were negative in five cases also prior to the initiation of the penicillin therapy may perhaps be due to a deficient culture technique.

A comparative study of the clinical course, the therapy and the post-mortem findings.

In improving cases of subacute bacterial endocarditis the signs of healing to be expected are, in addition to the disappearance of bacteria from the blood, a fall in temperature, a decrease of the sedimentation rate and a rise of the Hb level as well as gain in weight. In the cases published here it was not possible, however, to observe the disappearance of bacteria from the blood, since the blood cultures were negative before treatment, as already mentioned. It could be ascertained in all these cases that the temperature fell to normal on the second or third day of beginning treatment, but only one case showed a significant fall in the sedimentation rate, while the Hb level persisted low. In this case who died after an afebrile course of 8 weeks' duration of heart insufficiency, a histological process of improvement could also be demonstrated. No inflammatory cells were found in the valves which only revealed scar formations and verrucose nodes. Yet there were in this case as well as in two other, unmistakable bacterial colonies in the valves on the margin of the connective

tissue, but no cellular reactions in their vicinity. These findings contradict several post-mortem studies already published, where no bacteria were found in the valves. In fact the general opinion prevails that bacteria are destroyed under treatment (*Geiger 1947, Rosenblatt 1945, Thill 1947*). Nor is the process of recovery described by *Saphir 1946* supported by this material. *Saphir* found in the recovery stage necrosis, some bacteria and lime in the periphery of vegetations, i. e. on the free valvular margins. In the cases investigated here bacteria were invariably found in the basic fibrin layer on the border of the connective tissue.

Observations similar to those of this paper have, however, been made already (*Jones, Kennedy, Mokotoff, Virkkunen, Christie*). The bacteria were then partly regarded as dead, partly as such whose virulence had decreased. The decreased virulence at least also becomes evident on the basis of this material, since no cellular reactions were found in the vicinity of the bacterial colonies. The investigations carried out did not yield any full certainty as to whether the bacteria were dead, but it appears probable that this was not the case, whereas the virulence only was reduced. In order to clarify this problem the researches are being continued by making post-mortem cultures of vegetations immediately after death. It must be stated as well that in several cases verrucose proliferation of the valvular tissue continued during the time of treatment.

It must therefore be deduced on the basis of this material that although the treatment which varied between 3 and 45 million penicillin units eliminated the fever in every instance and brought about a histologically demonstrable improvement, yet it was not sufficient for a complete destruction of bacteria. In a patient who was administered 45 million units no bacteria were found in the valves, but in the wall of the patent ductus arteriosus.

With regard to the aggravating effect of penicillin medication on heart insufficiency it must be observed that of the six cases in this material, four showed symptoms indicating heart insufficiency before the initiation of the treatment, and in three instances it was leftsided. In two of them heart insufficiency was intensified during treatment to such extent, that the latter must be held partly responsible for the aggravation. This opinion is supported by the histological findings in these cases in so far as both exhibited scar shrinkage in the aortic valves. It must be noted, however, that scar shrinkage was also found in patients who did not reveal any clinical symptoms of heart insufficiency. It is further noteworthy that those two cases who had no heart insufficiency before the beginning of treatment, did not develop any while it was in progress, and that a slight heart insufficiency affecting one of the cases was improved under treatment, although this patient died later with symptoms of heart insufficiency, having suffered a relapse of fever in the absence of adequate treatment. Yet it is.

evident that the scar formations developing at the sites of lesion during penicillin treatment can promote heart insufficiency, if valvular damage was advanced before the beginning of the treatment. Under these circumstances the result of antibiotic treatment is questionable in cases where insufficiency is advanced at the moment the therapy is instituted and the febrile course has been prolonged.

The fact that heart insufficiency is such a frequent cause of death in penicillin-treated patients may be primarily due to the circumstance that the patients who die are those whose heart insufficiency is already advanced when they come for treatment, while others stay healthy at least apparently for a long time; secondly, as demonstrated by *Fiese* 1947, the penicillin medication prevents the patient from dying of emboli and generalised sepsis, thus allowing insufficiency time to develop in a damaged heart.

Aside from the scar formation of valvular lesions under penicillin treatment, the heart insufficiency is promoted by other causes as well. These are in the first instance myocardial changes and possibly continual verrucose valvulitis, to which attention is drawn by *Virkkunen* (1948). Changes in the myocardium are common in association with subacute bacterial endocarditis (*Saphir* 1946). Myocardial lesions then manifesting themselves are abscesses, diffuse or local inflammation, perivascular accumulations of round cells, small infarcts and Aschoff's bodies. In addition to them, *Saphir* describes in penicillin-treated cases special foreign body granulomas which, according to him, develop around the calcium emboli from improving valvular vegetations. Such granulomas were not found in this material. Myofibrosis was manifested in all the cases and in one who had active inflammation in the valves, it involved the myocardium as well.

As already mentioned, a possibly continuing verrucose valvulitis has also been ascribed some significance in promoting heart insufficiency in patients who have apparently recovered under penicillin therapy. In this material three cases who had no active valvular inflammation deeper in the valvular tissue, developed small subcutaneous nodes formed by granulation tissue. Their appearance can, as supposed by *Virkkunen*, be partly attributed to avirulent streptococci, particularly since all such cases had bacterial colonies in the valves, without any adjacent cellular reactions. Observations made in animal experiments also argue for this possibility. It has been experimentally demonstrated (*Clawson* 1945, *Dick et Schwartz* 1946) that even less virulent streptococci can produce endocarditis, often typical subacute bacterial endocarditis, provided the bacteria are administered for a sufficient length of time. It can therefore be assumed that streptococci detected in the heart of patients treated with relatively massive penicillin doses, maintain a continual slight verrucose valvulitis which is a cause of later developing heart insufficiency.

Summary.

The material comprises six unselected cases of subacute bacterial endocarditis with pronounced clinical symptoms, treated with penicillin and examined postmortally. Only one case had a positive blood culture. Two had a patent ductus arteriosus. Three revealed changes chiefly in the aortic and three chiefly in the mitral valve.

The observation is made that even if quite small quantities of penicillin often abate the fever and bring about an also histologically discernible improvement, yet even considerable amounts of penicillin are often insufficient for a complete destruction of the bacteria.

Streptococci continuing their existence after an insufficient penicillin medication, aside from being able to cause a relapse, probably also maintain a verrucose valvulitis which can later promote the appearance of heart insufficiency. However, it seems that the appearance of heart insufficiency is mainly affected by the scar formations at the sites of ulceration.

An early diagnosis is imperative if the treatment is to yield good results, since if a patient has already developed a severe insufficiency, the significance of an antibiotic treatment is questionable, the scar contractions developing in the process only aggravating the heart insufficiency which rapidly runs a fatal course.

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MORPHOLOGICAL CHANGES IN THE GASTRO- INTESTINAL TRACT OF THE WHITE RAT FOLLOWING INANITION*)

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During the European hunger endemic at the end of the second World War protracted and severe diarrhoeal diseases frequently occurred. In spite of the best possible conditions for ravishing epidemics, especially in the concentration camps, strangely enough only a rather small number of the numerous diarrhoeas were infectious. This is also confirmed, i. a. by Adelsberger (1946), Liscomp (1945), Thaysen & Thaysen (1945), Koning (1946), and Lamy, Lamotte & Lamotte-Barillon (1946).

A number of authors (Faroy, Arnous & Ricard, 1946; Aykroyd & Copalan, 1945; Edge, 1945) have assumed that most of the non-infectious diarrhoeas are due to a B-avitaminosis. It is not very likely, however, that avitaminosis would occur to any considerable extent owing to the heavily reduced caloric value of the food in the European hunger areas. This is experimentally proved by Keys (1946) who shows that human subjects living on European hunger rations for half a year do not present avitaminotic symptoms. A large majority of authors (i. a. Burger, Sandstead & Drummond (1945); Liscomp, 1945; Thaysen & Thaysen, 1945; Lamy, Lamotte & Lamotte Barillon, 1946, and Makomaski, 1946) also claim that avitaminoses were practically not observed clinically during the European hunger endemic, and that the B-vitamin complex did not affect the diarrhoeas. It is consequently unlikely that the diarrhoeas may have been of avitaminotic nature.

It is quite characteristic that the diarrhoeal disease primarily occurred among the most starved sections of population, and that its intensity in the individual patient was parallel to the universal atrophy of the organism. This is particularly claimed and born out by

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Perakis & Bakalos (1943); *Liscomp* (1945); *Makomaski* (1947) and *Thaysen & Thaysen* (1948). Accordingly it will be reasonable to consider the diarrhoeal disease as a link in the hunger disease in line with its additional characteristic symptoms: Cachexia, edema and polyuria. It has, therefore, been assumed that the diarrhoeal disease as well as the other symptoms mentioned are caused by the diet, insufficient in calories and protein. In the Anglo-Saxon literature the diarrhoea is frequently referred to as »nutritional diarrhoea«. The term »nutritional diarrhoea« must, however, in our opinion, be taken to mean diarrhoeal diseases caused by nutritional injuries of any kind whatever. The term will consequently include something else and more than the specific diarrhoeas due to starvation, (for instance also diarrhoea of pellagra). We have, therefore, similar to the terms hunger cachexia and hunger edema preferred the term »hunger diarrhoea«.

The hunger diarrhoea had a typical clinical course. The stools were profuse, watery or slightly feculent and frequently contained undigested food remnants. Occasionally they contained blood, but, as far as we know, never pus. The course was always afebrile in uncomplicated cases. Anorexia, nausea or vomiting were not characteristic, on the contrary, nearly all patients suffered from hunger and thirst. The consequent frequent break of diet always aggravated the diarrhoea, not unfrequently with fatal effect. The violent diarrhoeas often involved extreme exsiccation and acidosis. The hunger diarrhoea was, without any doubt, the most severe symptom of the hunger disease, and, at any rate the most frequent cause of death. The treatment should consist of a lenient diet during the first days, for instance with skim milk and thereafter rapidly proceeding to full diet rich in calories. Parenteral nutrition was rarely necessary and moreover always very risky (circulatory collapse).

The said clinical picture in connection with demonstration of undigested food in feces suggests a progressively reduced digestive and resorptive function of the gastro-intestinal tract in the course of chronic starvation. By autopsies *Perakis & Bakalos* (1943), *Liscomp* (1945), *Thaysen & Thaysen* (1945), *Makomaski* (1947), and *Pollack* (1947) have found atrophy of the tissue of the intestinal tract. Especially the mucosa has been affected, and particularly in the colon muscularis or even serosa has been found denuded on big parties.

Experimental investigations by *Jackson* (1915) show that the weight of the intestinal tract in rats decreases heavily during starvation. When the total weight of the rats is reduced by averagely 35 per cent, the weight of the gastro-intestinal tract, the liver, and the spleen will be reduced by about 57 per cent. The reduction in weight of muscles and integuments corresponds approximately to that of the whole of the organism, while the weight loss in nerve tissue, lungs, heart, kidneys, and a series of glands is less than the average. *Sum* (1927) and *d'Ancona* (1927) microscopically found atrophy of the

mucosa of the gastro-intestinal tract in starved rats and mice. The atrophy is most pronounced in the small intestine, especially in the duodenum and here especially hits apex of villi, while the basal coat of the mucosa keeps proportionally longer.

Cori & Cori (1927) found that the resorption of glucose is slower in rats having starved 48 hours than in rats having only starved 24 hours. *Magee & Reid* (1933) showed that the hyperglycemic and specific dynamic effect of glucose is reduced in starving fowls. Further it appeared that the same dose of glucose always gives diarrhoea in fowls having starved more than 96 hours, while it is absolutely retained and resorbed in fowls having starved less than 72 hours. *Magee & Reid* further found that the motoric function of the small intestine and especially the motility of villi is heavily reduced during starvation. Correspondingly is found (*Rumpel & Knack*, 1916 and *Pollack*, 1947) by X-ray examination of hunger diarrhoea patients typical «deficiency bowel pattern» expressive of reduced motoric function of the intestine in spite of the diarrhoeas. (The same X-ray picture is found at idiopathic steatorrhoea (Gee-Thaysen's disease) and at the African diarrhoeal hunger disease Kvashiorkorr.)

To summarize, the experiments with animals show that the intestine suffers surprisingly heavily during starvation. Clinically as well as on post mortem examination so many points of resemblance with the hunger diarrhoea in starving people are found that it may tempt to further extension of the experiments already made in this direction.

Material and Experimental Technique.

For the experiments is used a breed of rats of an average weight of 122 g (114—135 g). The animals are not fully grown up. Rats from this breed which, during the experiment, have had free access to food had an average increase in weight of 1.7 g in 24 hours. Rats of average weight consume about 25—30 g of the food used in 24 hours. (This food consists of a mixture of 20 kilos rolled wheat, 2 kilos powdered skim milk, 3 kilos casein and 1 kilo luncern flour together with a little sodium chloride and calcium carbonate.)

Each experimental animal has been placed in a separate cage provided in the bottom with wire netting allowing the excretes to fall outside the cage. All test animals were allowed to drink as much water as they liked. A number of control animals have had access to unlimited food during the whole period of experiment. The remainder of the animals have been on inanition, partly partial (i. e. 7—8 g food a day), by which they averagely lost 1/3 of their weight in about 8—10 days, partly total, by which they had a similar loss of weight in about 1 days. It has been tried to re-feed a few of the starved animals after a loss in weight of about 1/3. For this re-feeding there has been used ordinary rat food in slightly reduced doses or, during the first days,

a concentrate of skim milk, gradually increasing to ordinary food. The stools have been controlled every day during the experiments.

The animals were killed by neck stroke. As soon as the animals were killed, or possibly found dead, the gastro-intestinal tract, liver, kidneys, and heart were taken out for weighing. All superfluous tissue, as for instance mesentery fat has been carefully cut away, and by cautious rinsing the contents of the stomach and intestine has been removed before weighing. At the same time preparations have been taken from stomach, intestine, liver, kidneys and heart for microscopy*). The preparation of duodenum has been taken nearly 1 cm from the pylorus, of jejunum 8—10 cm from the pylorus and of ileum 8—10 cm from the ileocaecal junction. (Microscopical examination has not been carried out on animals found dead to avoid possible errors arising from autodigestion of the intestine.)

Experimental results.

In table 1 a series of particulars regarding experimental conditions and results is to be found. A summary of food conditions for each test animal is also given; further explanation is specified in the section »Material and experimental technique«. The weight of the animals at the beginning of the experiment, during the same (before eventual re-feeding), and at the end of the experiment is given in g., and eventual loss in weight in per cent of the original weight of the animals. The weight of the organs is given in g. with one decimal (more exact weighing of the humid and rinsed organs we have not been able to allow ourselves for the present). Corresponding to the weight of each organ is also indicated the relative weight, i. e. that percentage the organ in question amounts to of the total body weight at section. By comparing the corresponding relative weights of the control animals you can easily decide whether the loss in weight of the organ corresponds to the average loss in weight of the organism or, maybe, is bigger or less than this.

As is to be seen from the table the gastro-intestinal tract as well as the liver lose considerably in weight during starvation, as the loss of weight in these organs is relatively higher than that of the whole organism. In the control animals the gastro-intestinal tract amounts to about 8 per cent. of the body weight, while in starved animals it only amounts to 5.1 to 7.2 per cent. With respect to the liver the corresponding figures are about 4 per cent. and between 2.4 and 3.8 per cent.; kidneys and heart, it must be admitted, lose slightly in absolute weight, but the relative weight of these organs is ordinarily increased. Compared with the control animals the spleens in the

*) All preparations are formalin fixated within 10 minutes after the animals have been killed.

starved animals were extraordinarily small (*Andreasen*, 1943), (the spleens are not weighed).

Further it appears from the table that the animals are affected rather heavily during the starvation, but that, by the same relative loss in weight, the general condition is most affected after total inanition.

In a few animals the stools were thin and mucous but not visibly mixed with blood, or evil-smelling during starvation.

It has been tried to re-feed a few individual animals. All animals which at once got free access to ordinary food died, while animals, which exclusively had concentrate of skimmed milk the first days, rapidly recovered.

The descriptions of the autopsies speak for themselves; it is most astonishing to see how rapidly the intestine recovers its normal macroscopical appearance by successful re-feeding.

Figg. 1—4 show a selection of microphotographs (magnification 1:50) partly of normal duodenum, jejunum and ileum and partly of the same section of the intestine in rats having starved under varying conditions as specified in table 1. It is chiefly conspicuous that the epithelium on villi in the starved animals is practically lacking, and that the destruction of the epithelium frequently extends right down to the bottom of the crypts. At the same time the submucosa has shrunk considerably, and also the muscularis can be rather atrophic. There is no symptom of edema or congestion. Finally a number of pictures show how the intestine is almost perfectly regenerated in re-feed animals. Microscopy of the liver in starved animals show chiefly loss of cytoplasm within the individual cells, but no major microscopic changes are traceable here, no more than in the stomach and colon.

Discussion.

Like *Jackson* (1915) we have shown that the weight of the intestine is considerably reduced during starvation as the loss in weight is comparatively larger here than that of the whole organism. By microscopy we have found out like *Sun* (1927) and *d'Ancona* (1927) that the heavy loss in weight is due to an atrophy of all layers of the small intestine, especially of the mucosa and submucosa. The alterations mentioned are, at the same loss in weight, independent of the fact whether the animals have been starved acutely or more chronically. At acute starvation the heavy alterations are evident already after 4 days. As appears from the description of the autopsy the deposits of fat have been consumed by this time, consequently the animals have had to cover their basal metabolic rate and consumption of energy by body proteins (the animals are all very unquiet during the starvation). Therefore, it is quite likely that the atrophy of the intestine is caused by the deficiency in calories and primarily by the lack of protein. On

Table 1.

No.	Diet	Initial weight	Weight at time of refeeding		Weight at necropsy		Gastro intestinal tract	
			g.	Weight loss in per cent of initial weight	g.	Weight loss in per cent of initial weight	g.	Weight in per cent of body weight at necropsy
1.	Full diet (4 days)	135			138		11.1	8.0
2.	Full diet (4 days)	130			133		10.8	8.1
4.	Partial inanition (10 days)	117			80	32	5.3	6.6
5.	Partial inanition (8 days)	119			86	28	5.4	6.2
6.	Total inanition (4 days)	115			76	34	4.8	6.3
7.	Total inanition (4 days)	121			79	35	5.3	6.6
8.	Total inanition (4 days)	120			85	29	4.4	5.1
9.	Total inanition (4 days) followed by re-feeding on normal food (4 days)	114	77.5	32	71	38	5.1	7.1
10.	As 9.	128	90	28.7	83	35	6.0	7.2
11.	Total inanition (4 days). Refed on skim milk followed by increasing amounts of normal food	135	94	30.4	134		10.7	8.0
3.	Partial inanition (10 days). Refed on skim milk followed by increasing amounts of normal food. (8 days)	120	82	32	123		10.1	8.2

*) A: subcutaneous adipose tissue and muscles well developed. Gastro-intestinal tract of a light grey colour, non-transparent. Well-marked mucosal folds. Abundant fat in oment and mesentery.

B: subcutaneous adipose tissue almost perfectly lacking. Muscular tissue somewhat scanty. No clinical edema. Intestinal wall especially of small intestine and

Liver		Kidneys		Heart		Clinical picture	Necropsy description*)
g.	Weight in per cent of body weight at necropsy	g.	Weight in per cent of body weight at necropsy	g.	Weight in per cent of body weight at necropsy		
5.56	4.0	1.2	0.9	0.5	0.4	Completely normal	A
5.45	4.1	1.2	0.9	0.5	0.4	Completely normal	A
1.9	2.4	0.7	0.9	0.4	0.5	General condition rather bad. Stools scanty but normal	B
2.6	3.0	0.8	0.9	0.5	0.5	General condition rather bad the last day. Diarrhoea the last day.	B
2.9	3.8	0.8	1.0	0.4	0.5	General condition rather bad the last days. Stools scanty but normal	B
2.4	3.0	0.9	1.1	0.4	0.5	General condition very bad the last day. Stools scanty but normal. Found dead	B
2.6	3.1	0.8	0.9	0.4	0.5	General condition very bad the last day. Stools scanty but normal. Found dead	B
2.1	3.0	0.6	0.9	0.5	0.7	General condition very bad the last day. Stools scanty but normal. Does not recover during re-feeding. Found dead	B-1
2.4	2.9	1.0	1.2	0.5	0.5	General condition very bad the last day. Stools scanty but normal. Does not recover during re-feeding. Found dead	B-1
7.4	5.5	1.1	0.8	0.5	0.4	General condition very bad the last day of starvation. Stools liquid and mucous. Gets perfectly normal on re-feeding	A
5.15	4.1	1.0	0.8	0.5	0.4	General condition very bad the last days of starvation. Complete recovery during re-feeding. Stools normal	A

coecum very thin and cellophane-like transparent. Fat in oment and mesentery almost perfectly lacking apart from scanty »gelatinous« remnants.
 B-1: same appearance as B., but stomach, duodenum and jejunum stuffed with undigested food.

the other hand it is hardly conceivable that manifest avitaminoses should be capable of developing so quickly.

Regarding the microscopical picture of the mucosa it cannot be excluded that the almost universal lack of epithelium is artificially produced during fixation. On the other hand it is evident, that the loss of epithelium is only found in starved animals and that the remaining epithelium in these specimens is flattened and atrophic. A possible artificial lesion, in our opinion, must therefore be the consequence of intravital changes of the epithelium resulting in decreased resistance to the trauma of fixation.

It is evident that the different functions of the intestine must suffer heavily owing to such a considerable atrophy. It also appears that the starved animals do not stand re-feeding on an ordinary diet. The loss in weight continues during such an attempt at re-feeding, the decrease being, however, at a slightly reduced rate. The reduced rate of the loss in weight is presumably mainly due to retention of considerable quantities of food in the stomach and the upper part of the small intestine, though it cannot, on that basis, be excluded that a smaller part is resorbed. It is, however, worth mentioning that all the food which is in the gastro-intestinal tract is quite indigested. If the starved animals get concentrate of skimmed milk the first days, they soon recover perfectly. The heavily injured small intestine is consequently capable of treating and resorbing this food. As mentioned it is astonishing to see how quickly the intestine regenerates on skimmed milk and how perfect this regeneration is.

As mentioned in the introduction also in human beings with hunger diarrhoea atrophy in the tissue of the gastro-intestinal tract is found. The alterations correspond entirely to the experiments in animals referred to, aside from the fact that in human beings the colon is usually most severely affected.

To judge from our experiences (1948, 1949) from a large material the »hunger diarrhoea« occurs at the moment when the patients have lost 20—30 per cent. in weight. According to the aforesaid it is reasonable to assume, that already at this loss in weight, there is such a pronounced atrophy of the gastro-intestinal tract that the reduced digestive and resorptive function, on which the diarrhoea is dependent, can be explained hereby. On their liberation from the German concentration camps numerous patients with diarrhoea died from the full diet they were offered (compare our animal experiments). On the other hand even patients suffering seriously from diarrhoea with a loss in weight of about 50 per cent. frequently recovered quickly on concentrate of skimmed milk per os. On a later examination of about 800 former patients with hunger diarrhoea we have not been able to indicate any protracted disturbances in the intestinal function which could possibly originate from the sometimes severe atrophy of the gastro-intestinal tract which must undoubtedly once have existed.

As will be seen from the aforementioned there are extremely many points of resemblance between the »hunger diarrhoea« and the experiences from our experiments on animals. We therefore feel entitled to assume that the hunger diarrhoea is primarily the consequence of pronounced atrophy in the tissue of the gastro-intestinal tract, and that this atrophy is dependent on a calorically insufficient food and lack of protein.

On the other hand it is not possible for the time being to explain why this specific organ suffers so heavily from hunger. Whether special facts assert themselves with regard to the circulation is difficult to determine. Some authors (*Wergeland et al.* (1946) and *Lamy et al.* (1946)) feel inclined clinically to show a connection between edema and diarrhoea. A decreased rate of circulation, indicated in starved human beings by *Cardozo & Eggink* (1946), might possibly contribute somewhat towards producing the diarrhoea by giving congestion in the intestine. It is known that in other congestive conditions (for inst. heart insufficiency and shock) diarrhoea may occur simultaneously with congestive conditions being ascertainable in the portal region by autopsies and severe edematous, sometimes ulceratively atrophic changes of the intestine may be indicated. Most frequently, however, neither edema nor congestion in the intestine are to be found in patients suffering from typical »hunger diarrhoea«. Clinically we (1949) have not succeeded in proving a positive connection between diarrhoea and edema. During the investigations on rats no edema nor congestion in the intestines was found.

Summary.

Experimental studies carried out on rats show marked atrophy and seriously reduced digestive power of the intestinal tract following inanition. Clinically and on post-mortem examination many points of resemblance are found between these experiments and the »hunger diarrhoea« in chronically starved patients.

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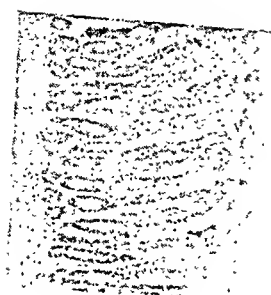
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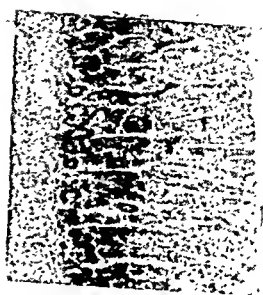
Duodenum.

Jejunum.

Ileum.



1 a

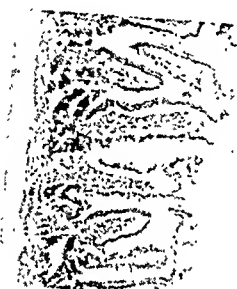


1 b



1 c

Small intestine of the rat (normal control).



2 a



2 b



2 c

Small intestine of the rat after total inanition (no. 6).

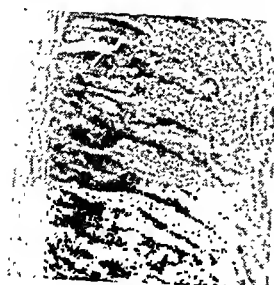


3 a



3 b

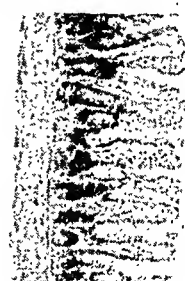
Small intestine of the rat after partial inanition (no. 4).



4 a



4 b



4 c

Small intestine of the rat after total inanition followed by refeeding (no. 11).

ON THE SEROLOGY OF THE KLEBSIELLA GROUP

By F. Kauffmann.

(Received for publication December 28th, 1948.)

The serological relations among *Escherichia*, *Aerobacter* and *Klebsiella* cultures, which in Bergey's Manual of Determinative Bacteriology (1948) constitute 3 different genera, hitherto have not been established, so that any reliable differential diagnosis thus far has not been possible. As the relations between *Aerobacter* and *Klebsiella* strains were particularly obscure, the purpose of the present work was to elucidate these questions.

A thorough review of the comprehensive literature cannot be given here and the reader is referred to the review given by Toenniesen, Julianelle, Edwards, Ellert & Gerkess, Goslings & Snijders, and Osterman & Rettger. The foundation for the serology of the *Klebsiella* group was laid by Toenniesen when he established that distinction must be made between the body or soma and the capsule and mucous envelope of the organisms in the Friedländer group. He was the first to demonstrate the carbohydrate nature of the capsule and emphasized its relationship to the virulence of the bacteria. Julianelle described 3 different capsule types in the Friedländer bacteria — A, B and C — besides the heterogeneous group X, all of which in the R form contained the same somatic antigen, a protein. Subsequently (1937) he stated that the *Klebsiella* group could be divided into 2 subgroups, on the basis of their R antigen, namely:

- I. All true Friedländer bacteria with capsules A, C and X.
- II. Rhinoscleroma, ozaena, granuloma and *lactis aerogenes* cultures.

P. R. Edwards found no fundamental difference between Friedländer and *lactis aerogenes* cultures, and he recommended that both species be reckoned as belonging to a common capsular group, stating: "lactis aerogenes is so closely related to the other encapsulated forms that they should be classified in the same genus. No constant differences have been observed which could be used to separate the organisms into two or more species". He investigated especially *Klebsiella* strains from metritis in mares and found them all belonging to capsule type B (Julianelle).

Ellert & Gerkess studied acapsular forms of Friedländer, rhinoscleroma, ozaena and *lactis aerogenes* cultures isolated from encapsulated strains through spontaneous dissociation. In contrast to Julianelle these authors set

up 3 groups on the basis of the somatic antigens: I. Rhinoscleroma; II. *Lactis aerogenes*; and III. Friedländer + ozaena.

Goslings & Sniijders arrived at results deviating from those obtained by Julianelle and the above-mentioned authors. They found 4 groups: I. Rhinoscleroma; II. Friedländer; III. Ozaena; and IV. *Lactis aerogenes* (a heterogeneous group). They confirmed the findings of Julianelle with regard to the three capsular types in the Friedländer strains (A, B and C) and they set up 3 different ozaena capsule types: D, E and F. In addition, they found that the capsule type common to all rhinoscleroma bacteria was identical with type C in the Friedländer bacteria.

Osterman & Rettger investigated especially the relation between the Friedländer and the aerogenes group and carried out also some serological studies on the somatic antigens though without performing absorption experiments. In conclusion of their paper they say: »It is concluded that valid criteria have not been established for the differentiation of organisms of the Friedländer and coli-aerogenes groups«.

As to other publications, reference is made to a review by Parr who advocated that all members of »coliform bacteria« (*E. coli*, *E. freundii*, *A. aerogenes*, *A. cloacae* and *Klebsiella*) should be placed in one genus. Also Borman, Stuart and Wheeler suggested that all these bacteria be included in one genus: »*Colobactrum*«.

With regard to capsular staining and swelling reaction the reader is referred to works by Etinger-Tulczynska and Klieneberger-Nobel.

The writer is greatly obliged to Dr. Klieneberger-Nobel for her performance of special capsular and mucous stainings in numerous strains.

Material Investigated.

Biochemical and serological studies have been carried out with the following strains:

A. Strains from Foreign Laboratories.

I. Klebsiella pneumoniae,

from the National Collection of Type Cultures, London.

1. No. 204 L. I. P. M. Probably original Friedländer strain. 1920. The strain occurs in the O form.
2. Type A (Julianelle) No. 5054.
3. Type B No. 5055.
4. Type C (Julianelle) No. 5056.
5. No. 5057. The strain occurs in the O form.

Strain No. 3279 Biberratte was rough and was not further investigated.
Strain No. 3263 Sheep Pneumonia does not belong to the *Klebsiella* group as it is motile, does not split adonitol and liquefies gelatin.

From the American Type Culture Collection, Washington.

6. Type A (Julianelle) Sc. No. 4208.
7. Type B (Julianelle) No. 7380.
8. Type B (Julianelle) E. No. 4209.
9. Type C No. 10273.
10. Group X, F 12. No. 4211.
Strain No. 4210 does not belong to the *Klebsiella* group, but to the *Escherichia* group.

II. *Klebsiella rhinoscleromatis*.

From the National Collection of Type Cultures, London.

11. No. 1936. E. P. Snijders, Amsterdam.
12. Type C No. 5046.
13. Type C No. 5047.
14. Type C No. 5048.
15. No. 5049 non-capsulated form, isolated from No. 5048.

From the Diagnosis Department of the State Serum Institute,
Copenhagen.

16. *Klebsiella rhinoscleromatis* No. 1383/35—36.

III. *Klebsiella ozaenae*.

From the National Collection of Type Cultures, London:

17. Type D No. 5050.
18. Type E No. 5051.
19. Type F No. 5052.
20. No. 5053 non-capsulated form, isolated from E 5051.

The strain No. 459 N. S. Ferry 110895 does not belong to the *Klebsiella* group, but to the *Proteus morgani* group.

From the Diagnosis Department of the State Serum Institute,
Copenhagen.

21. *Klebsiella ozaenae*.

IV. *Bacterium lactis aerogenes*.

From the National Collection of Type Cultures, London.

22. No. 243.
23. No. 418.
24. No. 4140.
25. An original *B. lactis aerogenes* strain of Escherich, from the Diagnosis Department of the State Serum Institute, Copenhagen; no typical *aerogenes* strain as it forms indole and liquefies gelatin.

B. *Own Material, isolated in Copenhagen.*

- I. 5 *Klebsiella* strains isolated from sputum:
Nos. 380, 915, 916, 1058 and 4631.
Nos. 380, 916 and 4631 were isolated 10 years ago, while Nos. 915 and 1058 are freshly isolated strains.
- II. 100 *Klebsiella* strains freshly isolated from urine from 99 cases, as in one case 2 different strains were isolated.

Thus a total of 130 strains of the *Klebsiella* group were examined and compared with test strains of the *Escherichia* group.

Biochemical Studies.

These examinations were carried out with culture media employed for differentiation of the *Escherichia* group. The capacity to decompose urea was tested on urea agar of Christensen and in the liquid medium of Ferguson & Hook. The reactions typical of *Escherichia* strains are reported in two preceding papers by Kauffmann (b)

and Kauffmann & Perch. From the comprehensive literature on the biochemical aspects of *Escherichia* and *Klebsiella* strains, it is evident that there is no reaction specific of either group, and that between the typical representatives of each group we meet with cultures forming a link between the two. In order to demonstrate the differences between the two groups the reactions of 10 typical cultures of *Escherichia* and *Klebsiella* are listed in Table 1.

Table 1.
Biochemical Behaviour of typical Escherichia- and Klebsiella-Strains.

	<i>Escherichia</i>					<i>Klebsiella</i>				
	1	2	3	4	5	1	2	3	4	5
Adonitol	—	—	—	—	—	+	+	+	+	+
Dulcitol	—	+	+	+	+	—	—	—	+	—
Sorbitol	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	—	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	—	—	+	+	+	+	+	+
Inositol	—	—	—	—	—	+	+	+	+	+
Lactose	+	+	+	+	+	×	+	×	+	+
Sucrose	—	—	—	+	+	+	+	+	+	+
Mannitol	++	++	++	++	++	++	++	+	++	++
Glucose	++	++	++	++	++	++	++	+	++	++
Indole	+	+	+	+	+	—	—	—	—	—
H ₂ S	—	—	—	—	—	—	—	—	—	—
Gelatin	—	—	—	—	—	—	—	—	—	—
Simmons' Glucose agar	+	+	+	+	+	×	+	—	+	+
Simmons' Citrate agar	—	—	—	—	—	—	+	—	+	+
KNO ₃	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	—	—	—	—	—	+	+	—	+	+
Methyl-red	+	+	+	+	+	—	—	+	—	—
Ureagar	—	—	—	—	—	+	+	—	+	+
Urea liquid	—	—	—	—	—	+	+	—	+	+

Key: *Escherichia* 1 = U 5/41, 2 = U 9/41, 3 = U 14/41, 4 = Bi 7509/41, 5 = Bi 316/42.

Klebsiella 1 = Friedländer 916, 2 = Friedländer B 5055, 3 = Rhinoscleroma C 5046, 4 = No. 919, 5 = No. 119.

Adonitol to Glucose: — = negative after 30 days, + = positive after 1 day, × = late and irregular.

Gelatin: — = negative after 60 days.

Simmons' agar, Voges-Proskauer, Methyl-red, Urea: — = negative after 4 days.

Considering first the upper part of Table 1, it is a striking feature that in contrast to *Escherichia* strains the *Klebsiella* strains promptly split nearly all the substances added to the media. It is possible only to differentiate the *Klebsiella* strains in dulcitol-positive and dulcitol-negative cultures. While 33 strains of capsule type 10 ferment dulcitol, 20 strains of type 8 and 40 strains of type 9 did not break down dulcitol. It must be emphasized in particular that adonitol and inositol are fermented by all the *Klebsiella* strains. In contrast hereto, adonitol is attacked only by about 10 % of the *Escherichia* strains, and inositol even less frequently. None of the *Klebsiella* strains form indole. While a majority of the *Klebsiella* strains grow on Simmons' citrate agar, a majority of the *Escherichia* strains give a negative reaction on this medium. The Voges-Proskauer reaction is nearly always negative for *Escherichia* strains, nearly always positive for *Klebsiella* strains. Conversely, the methyl-red reaction is usually positive for *Escherichia* strains, in contrast to *Klebsiella* strains. Hitherto no *Escherichia* strain has been found to give a positive urea reaction, whereas most *Klebsiella* strains proved capable of splitting urea. Furthermore as most *Escherichia* strains are motile, while all *Klebsiella* strains are non-motile, we have a number of characters by which the 2 groups may be differentiated.

Finally, it must be mentioned that a great majority of all recently isolated *Klebsiella* strains produce abundant slime and possess thermostabile capsules. In contrast hereto, only a minority of *Escherichia* strains have slimy growth and possess thermostabile capsules (= A antigen). On recording the more important differences between typical *Escherichia* and *Klebsiella* cultures we have the following schema:

<i>Typical behaviour of Strains of the</i>		
	<i>Escherichia group</i>	<i>Klebsiella group</i>
Adonitol	—	+
Inositol	—	+
Indole	+	—
Simmons' citrate agar	—	+
Voges-Proskauer	—	+
Methyl-red	+	—
Urea agar	—	+
Motility	+	—

So if we are dealing with non-motile, slime producing, Gram-negative rods which split adonitol and inositol but form no indole, they are most likely to be members of the *Klebsiella* group. Slimy *Escherichia* strains as a rule form indole and are not able to split adonitol and inositol.

In the following a comparison will be made between the cultural aspects of 100 *Klebsiella* strains freshly isolated from urine and 100 *Escherichia* cultures, the cultural aspects of which have been reported already in the paper of Kauffmann & Perch just mentioned.

Cultural Behaviour of

	100 <i>Escherichia</i> strains	100 <i>Klebsiella</i> strains
Adonitol:	11 +, 1 —+, 88 —	100 +
Dulcitol:	40 +, 28 —+, 32 —	33 +, 67 —
Sorbitol:	96 +, 3 —+, 1 —	100 +
Arabinose:	100 +	100 +
Xylose:	94 +, 5 —+, 1 —	100 +
Rhamnose:	85 +, 11 —+, 4 —	93 +, 7 —+
Maltose:	100 +	100 +
Salicin:	12 +, 67 —+, 21 —	100 +
Inositol:	1 +, 2 —+, 97 —	98 +, 2 —+
Lactose:	100 +	99 +, 1 —+
Sucrose:	22+, 15 —+, 63 —	99 +, 1 —+
Mannitol:	100 +	100 +
Glucose:	100 +	100 +
Gas production:	99 +, 1 —	100 +
Indole:	92 +, 8 —	100 —
H ₂ S:	4 +, 96 —	100 —
Gelatin:	100 —	100 —
Simmons' Glucose:	100 +	99 +, 1 —
Simmons' Citrate:	3 +, 97 —	98 +, 2 —
KNO ₃ :	100 +	100 +
Voges-Proskauer:	100 —	91 +, 9 —
Methyl-red:	100 +	7 +, 93 —
Urea agar:	100 —	99 +, 1 —

Key: For simplification we have designated in the above scheme a positive reaction after 24 hours with +, a delayed positive reaction with —+, and a negative reaction with —. Thus 11 +, 1 —+, 88 — mean that 11 strains were promptly positive, 1 strain fermented the medium late, and 88 strains gave a negative reaction. Failing gas formation, which was tested only in glucose and mannitol, was decided after 4 days observation, as also the absence of growth on Simmons' glucose and citrate agar. Failure to form indole was observed in 2 tubes tested after 1 and 2 days. The KNO₃, Voges-Proskauer, methyl-red and urea reactions were read after 4 days.

The 100 *Klebsiella* strains mentioned in the survey above were freshly isolated from urine and found to belong chiefly to capsule types 8, 9 and 10. All the 33 cultures splitting dulcitol belonged to type 10. The late fermentations of rhamnose, inositol, lactose, and sucrose involved merely a slight delay of 2—4 days.

On summing up the results, then, it may be said that *Klebsiella* strains freshly isolated from urine are able to split the following substances: adonitol, sorbitol, arabinose, xylose, rhamnose, maltose, salicin, inositol, lactose, sucrose, mannitol and glucose. The capacity for gas production, which was tested only in mannitol and glucose, was present in all cultures.

No indole or H₂S was found, and gelatin was not liquefied. Most of the strains grew on Simmons' agar with glucose and citrate and gave a positive KNO₃ and Voges-Proskauer reaction. As a rule, the methyl-red reaction was negative, while urea usually was split.

So here we are dealing with characteristic biochemical properties, distinctly deviating from those encountered in the *Escherichia* group.

While most of the cultures examined by the author belong to the *Escherichia* group or to the *Klebsiella* group, various strains occupy an intermediary position. The latter category includes, for instance, an original »*B. laetis aerogenes*« from *Escherich* which Vahlne has described as belonging to the *Escherichia* O group 9. It was demonstrated that this strain formed indole and slowly liquefied gelatin and thus was no typical »*aerogenes*« strain. The same applies to some other strains which here will be mentioned but briefly.

Just as the *Salmonella* group also includes indole-forming and gelatin-liquefying strains, cultures with these properties occur also in the *Klebsiella* group. Some cultures, including strains from the National Collection of Type Cultures in London designated as *B. cloacae*, form no indole but liquefy gelatin.

So far we have been dealing with differentiation of the *Klebsiella* group from the *Escherichia* group, now we will turn to the cultural differentiation within the *Klebsiella* group. For this purpose the following substances or reactions are especially useful: duleitol, urea, organic acids, particularly d-tartrate, sodium-citrate, mucate, and the Voges-Proskauer reaction. Some characteristic biochemical types are presented in Table 2.

From Table 2 it is evident that duleitol is split by 2 types, 1:3 and 1:10, whereas the other types are unable to break down this alcohol. The more frequent occurring capsular types 8, 9 and 10 also may be differentiated culturally by means of duleitol and sodium-citrate (tested in fluid bacto-peptone medium).

	Duleitol	Sodium-citrate
Type 8	—	+
» 9	—	—
» 10	+	—

As reported in the literature, the rhinoscleroma bacteria occupy a special position. They are unable to decompose urea and organic acids; and they fail to grow on Simmons' glucose and sodium-citrate agar.

On summing up the above it may be said that by means of biochemical methods we are able to decide whether a strain belongs to the *Escherichia* group or to the *Klebsiella* group. Furthermore, it is possible by means of certain substances to set up various biochemical types within the *Klebsiella* group.

With a view to the frequent occurrence of hemolytic and hem-agglutinative strains in the *Escherichia* group, 58 *Klebsiella* strains belonging to various types were examined for these characters. The tests for hemolysis were carried out with employment of washed horse erythrocytes in 1 % peptone solution, the tests for hemagglutination

Table 2.
Biochemical Differentialdiagnosis.

Strains	Formula	Dul- citol	Urea	Organic acids			V. P.	Num- ber of strains
				d	Ci.	Mu.		
A 4208 . . .	1 : 1	—	+	+ ²	+ ²	+ ¹	—	3
A 916 . . .	1 : 1	—	+	+ ²	+ ²	+ ¹	+	1
B 5055 . . .	1 : 2	—	+	+ ¹	+ ³	+ ³	+	1
B 243 . . .	1 : 2	—	+	—	+ ¹	+ ¹	+	2
C 5056 . . .	1 : 3	+	+	—	+ ¹	+ ¹	—	3
B 7380 . . .	2 : 2	—	+	—	—	+ ¹	—	2
B 4631 . . .	2 : 2	—	+	—	+ ¹	+ ¹	+	1
Rhin. C 5046 . . .	2 : 3	—	—	—	—	—	—	6
Oz. D 5050 . . .	2 : 4	—	+	+ ²	—	+ ¹	—	1
Oz. E 5051 . . .	2 : 2	—	—	+ ²	+ ²	+ ¹	—	1
Oz. F 5052 . . .	2 : 6	—	—	+ ³	—	+ ⁷	—	1
Aer. 4140 . . .	1 : 7	—	+	+ ¹	+ ¹	+ ¹	+	1
No. 1015 . . .	1 : 8	—	+	—	+ ¹	+ ¹	+	19
" 56 . . .	: 9	—	+	—	—	+ ¹	+	27
" 919 . . .	1 : 10	+	+	—	—	+ ¹	+	26
" 390 . . .	3 : 11	—	+	—	—	+ ¹	+	2
" 313 . . .	1 : 12	—	+	+ ¹	+ ¹	+ ¹	+	1
" 1470 . . .	: 13	—	+	+ ¹	—	+ ¹	+	1
" 1193 . . .	: 14	—	+	—	—	+ ¹	+	1
Total								100

Key: Organic acids in bacto-peptone solution: d = d-tartrate, Ci. = sodium-citrate, Mu. = mucate. V. P. = Voges-Proskauer. Rhin. = Rhinoscleroma, Oz. = Ozaena, Aer. = Aerogenes, No. = Number.

with guinea-pig erythrocytes (10 %). None of the Klebsiella strains examined were hemolytic, while 2 strains, an old laboratory strain 204 from the Lister Institute and a freshly isolated type 10 strain from urine, produced hemagglutination. Thus Klebsiella strains are non-hemolytic and as a rule non-hemagglutinative, behaving in these respects as Escherichia strains of O groups 8 and 9.

Serological Studies.

Before turning to the determination of the individual antigens, it will be appropriate to make some general remarks concerning the serology of the Klebsiella group. In contrast to the view maintained by Julianelle, the serological features of this group are dependent not only upon two factors — the capsules and the somatic R antigens — but also on the O antigen. More correctly, it is even a matter of four

factors, namely: the mucoid envelope, the capsule, the O antigen, and the R antigen. Probably, however, the mucoid envelope is serologically identical with the capsule, even though this has not hitherto been proved experimentally. At any rate we have to distinguish between the following three factors:

1. Capsular antigen = *K antigen*,
2. Somatic smooth antigen = *O antigen*, and
3. Somatic rough antigen = *R antigen*.

For the serological determination of the types the two first antigens are decisive. While the mucoid envelope, capsule, and O antigen are carbohydrate compounds, the R antigen, according to Julianelle, is protein in character. As these antigens may appear in various combinations, the prevailing nomenclature (M = mucoid form, S = smooth form, and R = rough form) is not sufficient for the designation of all the forms encountered.

Besides, some authors have employed these symbols in different senses, and therefore it seems preferable to designate the forms after their antigens in the following way:

I. Smooth Forms:

1. MKO form = mucoid, capsular, with O antigen.
2. KO form = non-mucoid, capsular, with O antigen.
3. MO form = mucoid, acapsular, with O antigen.
4. O form = non-mucoid, acapsular, with O antigen.

II. Rough Forms:

1. MKR form = mucoid, capsular, without O antigen.
2. KR form = non-mucoid, capsular, without O antigen.
3. MR form = mucoid, acapsular, without O antigen.
4. R form = non-mucoid, acapsular, without O antigen.

In the presence of full development of the O antigens the R antigen occurring in all smooth forms will not make itself manifest in agglutination tests, and in immunizing experiments it will have only a weak antigenic effect. So, for the sake of simplification it will be convenient to leave the symbol R out in the designations of the smooth forms I_1 — I_4 , but its presence in all these forms has to be realized and kept in mind.

Although *Klebsiella* cultures with fully developed K antigen are O and R inagglutinable in tube agglutination tests (20 hours at 50° C.) not only capsular agglutination but also O or R agglutination may occur. For the production of pure capsular agglutination, therefore, it is necessary to absorb the sera with O and R forms.

These difficulties may be avoided by employment of the capsular-

swelling reaction as this permits one easily to distinguish between capsular swelling with agglutination and agglutination without capsular swelling. Therefore, for the demonstration of capsular type, the capsular swelling test is to be recommended.

Determination of O Antigens.

Determination of O antigens can be carried out with sera produced with capsular cultures, if acapsular bacteria are used for the agglutination. If we wish to use pure O sera, however, these immune sera must be produced with acapsular bacteria. The tube agglutination test may be carried out with a formalin-treated broth culture or with a boiled broth culture of acapsular strains. Determination of O antigens also may be carried out with suspensions of living acapsular forms from agar cultures by the slide agglutination method.

For a more thorough investigation of the O antigens sera were produced with capsular as well as acapsular cultures using both formalin-killed broth cultures and boiled broth cultures (2½ hours at 100°). Thus for a number of strains 4 different sera were available, each of which was tested both with formalin-killed and boiled acapsular cultures. Two rabbits were used to produce each serum. Their sera were mixed in order to exclude accidental phenomena. Altogether 133 rabbit immune sera were used to carry through the present serological investigation (determination of O and capsular antigens). For this reason alone it would be impossible to give a complete account of all the results.

In order to obtain acapsular cultures, continuous passages were carried on in 50 % bile broth, transfers being made once or twice every week. Not in every instance, however, could acapsular forms be obtained.

On the basis of the O antigens, the *Klebsiella* cultures examined could be divided into three O groups (1, 2 and 3). O group 1 is characterized by its close serological relationship to the *Escherichia* O group 19. In the previous work of Kauffmann (c) it was established, that O group 19 is made up of 2 different subgroups, as the test strain for this group (8188/41) possesses O antigen not present in strain 8858/41. This was expressed by giving strain 8188 the formula 19+, strain 8858 formula 19. For the sake of clarity we will now give the antigenic formula for strain 8188 as 19a, 19b and the formula for 8858 as 19a.

All *Klebsiella* strains of O group 1 in acapsulated state are agglutinated strongly by *Escherichia* serum 19a, 19b. Conversely, all sera produced with *Klebsiella* strains of O group 1 agglutinate *Escherichia* strain 8188 in a markedly granular form, whereas strain 8858 is not agglutinated by these sera. The outcome of some agglutination experiments within *Klebsiella* O group 1 is recorded in Table 3.

Table 3.
Results of Tube Agglutination.

Strain	O group	Sera						
		Klebsiella			Escherichia		Klebsiella	
		204	5056	418	8188	8858	4209	5053
K 204	1	5120	2560	2560	1280	0	40	20
K 5056	1	5120	2560	2560	640	0	40	20
K 418	1	5120	2560	5120	640	0	40	20
E 8188	19	5120	2560	5120	5120	2560	40	20
E 8858	19	0	0	0	2560	5120	0	0
K 4209	2 A	40	40	80	0	0	2560	640
K 5053	2 B	0	0	0	0	0	640	5120

Key: Strain K = Klebsiella, E = Escherichia (= Coli).

The Klebsiella sera were prepared by formalinized broth culture of O forms, the Escherichia sera by boiled broth culture. The cultures used for agglutination tests were the same as those employed for immunization. The agglutination was read after 20 hours in waterbath at 50° C. The numbers state the titre, 0 means negative in dilution: 1:20.

Through cross-absorption experiments it could be demonstrated that the Klebsiella O 1 antigen is identical with the Escherichia 19b antigen. Escherichia strain 8188 was able completely to exhaust O sera of the Klebsiella O group 1. Conversely, Klebsiella strains of this group were able completely to absorb Escherichia 19a, 19b serum only when the 19a agglutinin had been removed beforehand by absorption with strain 8858.

Table 4.
Results of Absorption.

Strain	E 8188				K 204	
	not absorbed	- absorbed by			not absorbed	absorbed by E 8188
		E 8858	K 204	8858 + 204		
E 8188	5120	1280	5120	0	5120	0
E 8858	2560	0	2560	0	0	0
K 204	1280	1280	0	0	5120	0

Key: E = Escherichia

K = Klebsiella

O = negative in dilution 1:40

From Table 4 it is evident that the 19b titer for Escherichia 8188 serum is 1:1280, while the 19a titer is between 1:2560 and 1:5120. In absorption experiments the Escherichia strain 8858 removes only the 19a agglutinin, while Klebsiella strain 204 removes only the 19b agglutinin. Serum produced with Escherichia strain 8188 may be ex-

hausted completely by combined absorption with *Escherichia* 8858 + *Klebsiella* 204.

Determination of *Klebsiella* group 1 may therefore be carried out easily by means of an *Escherichia* 19a, 19b serum — that is, if acapsular strains possessing the O antigen are at disposal. If only mucoid capsular strains (MKO form) are available, it is necessary first to produce immune sera in order to ascertain whether these sera agglutinate the *Escherichia* strain 8188. In this case it makes no difference whether a formalin-killed or a boiled broth culture is used for immunization, as O antibodies will be found in either case. All sera agglutinating *Escherichia* strain 8188 belong to O group 1, whereas all sera incapable of agglutinating this strain belong to other groups, unless they are R sera.

Between O group 1 and O group 2 there is only very slight, if any, O-antigenic relationship. The weakest overlapping reactions are obtained on employment of sera produced with boiled broth culture of the MKO form and by carrying out the agglutination test with boiled O forms (see Table 5).

Table 5.
Results of Tube Agglutination in O Groups 1, 2A and 2B.

Strain	Sera				
	O group	<i>Klebsiella</i> Group 1 4208	<i>Klebsiella</i> Group 2A 4209	<i>Klebsiella</i> Group 2B 5050	<i>Escherichia</i> Group 19 8188
K 204	1	5120	40	20	1280
K 380		2560	20	0	1280
K 41		2560	20	0	640
K 4631	2 A	20	1280	0	40
K 7380		40	2560	20	0
K 4209		40	5120	40	0
K 5050	2 B	0	80	2560	0
K 5052		0	160	5120	0
K 5053		0	160	2560	0
E 8188	19	5120	20	0	5120

Key: The *Klebsiella* sera employed were prepared by boiled broth culture of MKO forms, and the *Klebsiella* cultures were boiled broth culture of O forms. The agglutination was read after 20 hours in waterbath at 50° C. 0 = negative in dilution 1:20.

While the conditions within O group 1 are very clear and indisputable, the O antigenic analysis of O group 2 has been given considerable difficulty and as yet the results are not conclusive. This group falls in 2 different subgroups, designated as 2 A and 2 B. The difference between these 2 subgroups is not always distinct in agglutination experiments, as some sera give strong cross-reactions.

By employing other sera, however, we get highly group-specific reactions suggestive of 2 different O groups. The matter is further complicated by the fact that each of the two subgroups (2 A and 2 B) is not uniform but consists at least of two different subdivisions, the antigenic relations of which might be expressed as follows:

Group 2 A = 2 a, 2 b

„ „ = 2 a

Group 2 B = 2 a, 2 c

„ „ = (2 a), 2 c, 2 d

Sera of group 2 A with O agglutinin 2 a are absorbed completely by strains of O group 2 B with the formula 2 a, 2 c, but not by a strain with the formula (2 a), 2 c, 2 d as this lacks a part of antigen 2 a.

Agglutinatively and antigenically antigen 2 a is dominant in group 2 A, whereas antigen 2 c is dominant in group 2 B.

In Table 6 several sera of group 2 are entered in order to show how differently they react.

Table 6.
Results of Tube Agglutination with Sera of O Group 2.

Strain	O Group	Sera					
		Group 2 A			Group 2 B		
		Fo. 7380	Fo. 5049	7380	Fo. 5050	5051	Fo. 5052
4631	2 A	6400	3200	1600	20	800	40
7380		6400	6400	800	40	800	40
5050	2 B	1600	800	40	12800	12800	6400
5053		1600	1600	40	25600	12800	6400
204	1	80	80	80	40	40	0

Key: The sera labelled Fo. were prepared by formalinized broth culture of O forms, the sera only indicated by numbers with boiled broth culture of MKO forms. The cultures used for agglutination tests were boiled broth culture of O forms.

By the use of the group-specific sera it is practicable even by means of slide agglutination to make the group diagnosis. So if we wish to obtain a very strong O agglutination with all strains of O group 2, we have to use two different sera (2 a and 2 a, 2 c) or a mixed serum in which all the cultures in this group are agglutinated strongly.

In 2 strains (390 and 674), belonging to capsular type 11, it was possible to demonstrate the O antigen of Escherichia O group 9, which in the Klebsiella group was designated as O 3. The identity of the two O antigens was demonstrated by cross-absorption.

In the Klebsiella group the O antigens cannot be demonstrated in

all the cases as many strains appear in the R form. This fact is ascertained not only on examination with acapsular strains but also on analysis of sera produced with capsular cultures. Thus, out of 90 K sera only 52 contained O antibodies — as is evident from Table 7. Of 14 K sera of type 8, 11 sera belonged to O group 1, and 1 serum to O group 2, while 2 sera contained no O agglutinins. R forms were particularly frequent in capsular type 9, as all 24 sera contained no O antibodies. So far, the writer's results indicate that different R antigens occur in the Klebsiella group. But here we will not enter further into this point as no systematic investigation concerning this question has been done.

Table 7.
Frequency of O and R Forms.

Capsule type	Number of K sera	Number of O groups			Number of R forms
		1	2	3	
1	4	4	0	0	0
2	7	3	4	0	0
3	8	3	5	0	0
4	1	0	1	0	0
5	1	0	1	0	0
6	2	0	2	0	0
7	1	1	0	0	0
8	14	11	1	0	2
9	24	0	0	0	24
10	23	13	0	0	10
11	2	0	0	2	0
12	1	1	0	0	0
13	1	0	0	0	1
14	1	0	0	0	1
Total	90	36	14	2	38

From the two above-mentioned strains of type 8 without O antigen acapsular cultures were isolated and used for the production of immune sera. These sera too were found to contain no O agglutinin. Thus it is evident that the capsular antigens do not prevent the formation of O antibodies.

The capsular form of a strain (aerogenes 4140) gave O agglutinins, whereas the acapsular form did not produce O agglutinins. Thus, on cultivation of acapsular forms also the O antigen may be lost together with the capsule.

In a majority of the cases in which R forms occur, the O antigen is supposed to have been lost in the human organism. The occurrence of R forms is considerably more frequent in Klebsiella strains freshly isolated from urine than in Escherichia strains. It must be emphasized, however, that all the Klebsiella pneumoniae strains of Julianelle con-

tained O antigens, so that Julianelle really had O antigens at his disposal without demonstrating their presence.

Immune sera were produced with 10 *Klebsiella* strains, using both the capsular and the acapsular forms. All 20 sera contained O agglutinins, thus demonstrating that as a rule the O antigen is not lost on isolation of acapsular forms. These strains included both original cultures from Julianelle and freshly isolated cultures.

As it might be of interest to know the O group relationship of the original *Klebsiella* strains obtained from other collections, it will be appropriate to tabulate them as follows:

O group 1:

Friedländer A 4208, 204, A 5054, 5057, B 5055, C 5056, C 10273, and X 4211.

Aerogenes 418, 243 and 4140.

O group 2 A:

Friedländer B 4209, B 7380 and 4631.

Rhinoscleroma C 5046, C 5047, C 5048, 5049, 1936 and 1383.

Strain B 4209 and all rhinoscleroma cultures contain antigen 2 a, while strains B 7380 and 4631 contain antigens 2 a, 2 b.

O group 2 B:

Ozaena D 5050, E 5051, 5053, F 5052 and ozaena Diagnosis Department. Strain D 5050 has the formula (2 a), 2 c, 2 d, while the formula of the other ozaena strains is 2 a, 2 c.

From this tabulation it is evident that all the older *Klebsiella* strains contain O antigens, and thus the R forms were found only in strains recently isolated from urine. An exception to this rule is found in strain 3279 from the Lister Institute that was completely rough, and no particular serological examination of it was made. In this connection one should refer to recent work by O. Möller who demonstrated the presence of two different R antigens within *Escherichia* O group 8 and 9. As briefly mentioned above, there is evidence to the effect that serologically different R antigens occur also in the *Klebsiella* group.

Here is a gap in our knowledge of the serology not only of the *Klebsiella* group but also of all other groups within the *Enterobacteriaceae*. It is obvious that a complete antigenic schema for these groups should comprise not only the O, K and H antigens, but also the R antigens. Determination of the R antigens is not of practical significance in type determination, and our diagnostic antigenic schemes hitherto set up are not in need of correction.

Determination of Capsular Antigens.

»Capsular sera« were produced by injection of 6-hour broth cultures killed by addition of 0.5 % formalin. The agglutination was carried out by the slide method with living agar culture as well as by the tube method with formalin-killed broth culture. In the slide test the sera were undiluted, or diluted in order to avoid overlapping reactions. In tube tests serial dilutions beginning at 1:2 were employed. The agglutination was read after the tubes had been left in water bath at 50° for 20 hours. A typical capsular agglutination is disk-shaped and easy to differentiate from the granular O or R agglutination. The capsular titers were between 1:16 and 1:128.

As in the capsules of the *Klebsiella* group we are dealing with thermostabile antigens, capsular antibodies may occur also in sera produced with boiled broth cultures (2½ hours at 100°). The capsular titers obtained in this way are very low, however, if they are present at all. On the other hand, broth cultures heated to 100° for 2½ hours gave a good capsular agglutination when tested with capsular sera produced with formalin-killed broth cultures.

O or R agglutination will occur in undiluted or diluted capsular sera in many cases, since not all the bacteria in a mass culture possess well-developed capsules and therefore are not O or R inagglutinable. Even though it is possible with some training to distinguish a capsular agglutination from an O or R agglutination, the *capsular swelling reaction* is preferable as the test of choice. The test is carried out with undiluted or diluted serum and a 5—6 hours broth culture, living or killed with 0.5 % formalin. In this way it is possible to demonstrate microscopically the occurrence of capsular swelling and thus to determine the type. In order to obtain type-specific reactions, in certain cases it is necessary to absorb the capsular sera with another strain with capsular relation to the serum concerned. As such overlapping reactions as a rule have a very low titer, it will suffice to dilute the serum instead of absorbing it.

If the agglutination is to be employed for type determination, it is recommended that it be done with pure capsular sera, i. e. sera absorbed with acapsular forms possessing the corresponding O or R antigens. It should be pointed out that the capsular titer is not always the same, since it is influenced by a more or less intense development of the capsule or mucoid substance. The more intense the development of the capsule or mucus, i. e., the more pronounced the antibody binding, the lower is the titer. For practical use the various sera have to be adjusted by dilution or absorption in such a way that they give a strong capsular swelling only with the homologous type. If this condition is met, only one microscopic examination is required — with one loopful of serum and one loopful of broth culture.

In keeping with the reports in the literature, it proved possible without difficulty to demonstrate capsular antigens A, B, C, D and F.

But thus far it has not been possible to demonstrate the E capsule in the ozaena bacteria. The only E strain at our disposal — strain E 5051 — formed mucus, it is true, but no capsule (confirmed by E. Klieneberger-Nobel through capsular staining), and thus this culture appeared in the above-mentioned MO form. Immunisation with a formalinized broth culture of this strain gave a serum which, besides O agglutinins, contained also M agglutinins (mucus-antibodies). Even though we thus were unable to confirm the occurrence of a special E capsule, the symbol E or number five was reserved for this K antigen, as the possibility that strains of this type, possessing a capsule may be found in other collections, must be considered.

Besides the previously described K antigens (A, B, C, D, and F), 8 new capsular antigens (7, 8, 9, 10, 11, 12, 13, and 14) were demonstrated in the present investigation. Of these antigens, 7 represents the capsule of the *lactis aerogenes* strain 4140, while 8—14 belong to various strains freshly isolated from urine. Since examination of a larger material may disclose the existence of additional new capsular antigens, it is suggested that they not be designated by means of capital letters but with arabic numerals as has been done in the *Escherichia* group. Therefore, the following capsular nomenclature is recommended:

Capsules already known:

A = 1, B = 2, C = 3, D = 4, F = 6. (As to E = 5, see above).

New capsules:

7, 8, 9, 10, 11, 12, 13, 14, etc.

Table 8 gives the outcome of tube agglutination experiments with capsular sera and formalin-killed broth cultures. Coincident O or R agglutinations are omitted.

As is evident from Table 8, among the various capsular types we meet with antigenic relations due to common K partial antigens. These overlapping agglutinations as a rule have only a low titer and usually they are not noticeable when the capsular swelling reaction is used. Also such overlapping reactions are absent in the sera that have only a low capsular titer. Thus, regular demonstration of these reactions is not to be expected. As is known (Avery, Heidelberger & Goebel) there is antigenic relationship between *Klebsiella* capsular type 2 (= B) and pneumococcus type 2 that is due to the existence of a partial antigen. In addition, antigenic relationship has now been established between *Klebsiella* types 8 and 9 and pneumococcus type 2 (see Table 8).

Considering the thermostability of the *Klebsiella* capsules, it seemed desirable to determine whether the same capsular antigens occurred also among the A antigens of the *Escherichia* group. Indeed, this proved to be the case, as *Klebsiella* capsule 10 is almost identical with *Escherichia* capsule 39. Further there is antigenic relationship between *Klebsiella* capsule 7 and *Escherichia* capsule 55, between *Klebsiella* capsule 8 and *Escherichia* capsule 34, and between *Klebsiella* capsule 11 and

Table 8.
Results of Tube Agglutination.
(Capsule Determination).

Cap- sule type	K-Sera														Pn. 2	Ear- lier desig- nation
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	64	0	0	0	0	4	4	0	0	0	0	0	0	0	0	A
2	0	128	0	0	0	0	0	8	4	0	0	0	8	2	4	B
3	0	0	128	0	0	0	0	0	0	0	0	0	0	0	0	C
4	0	0	0	32	0	0	0	0	0	0	0	0	0	0	0	D
5*)	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	E
6	8	0	0	0	0	32	8	0	0	0	0	0	0	2	0	F
7	2	0	0	0	0	4	64	0	0	8	0	0	0	0	0	.
8	0	4	0	0	0	0	0	128	4	0	0	0	0	0	0	.
9	0	2	0	0	0	0	0	8	128	0	0	0	0	0	0	.
10	0	0	0	0	0	0	4	0	0	64	0	0	0	0	0	.
11	0	0	0	0	0	0	0	0	0	0	64	0	0	0	0	.
12	0	0	0	0	0	0	0	0	0	0	0	32	4	0	0	.
13	0	0	0	0	0	0	0	0	0	0	0	(2)	32	0	0	.
14	0	0	0	0	0	0	0	0	0	0	0	0	0	64	0	.
Pn. 2	0	8	0	0	0	0	0	4	2	0	0	0	0	0	256	.

Key: *) = this strain Ozaena E 5051 has no capsule, but only slime.

Pn. 2 = Pneumococcus type 2.

0 = negative in dilution 1:2.

Escherichia capsule 37, i. e., these strains have partial antigens in common. It is very likely, therefore, that demonstration of new Klebsiella or Escherichia capsules will reveal other antigenic relations or capsular identity.

For the practical determination of types the capsular-swelling reaction is the test of choice. The results of this reaction with 6 frequently occurring types are recorded in Table 9.

Table 9.
Capsular Swelling.

Capsule Type	K-Sera					
	1	2	3	8	9	10
1	+	—	—	—	—	—
2	—	+	—	—	—	—
3	—	—	+	—	—	—
8	—	—	—	+	—	—
9	—	—	—	—	+	—
10	—	—	—	—	—	+

Table 9 shows that the type diagnosis can be made rapidly and definitely by means of the capsular-swelling reaction, so that this method is preferable.

Table 10 shows how often the individual capsular types were found among the *Klebsiella* strains isolated from urine. It will be noticed that 3 types — 8, 9 and 10 — comprise more than 90 % of all the strains. The strains entered in Table 10 were isolated from 99 patients with urinary infections, 2 types (8 + 9) being found simultaneously in one case.

Table 10.
Frequency of Capsule Types of Urinary Strains.

Capsule Type	Number of strains
2	1
8	20
9	40
10	33
11	2
12	1
13	2
14	1
Total	100

The type distribution in the total material is presented schematically in the following survey:

Type 1: Friedländer A 4208, A 5054, No. 380, 916 and 1058. All isolated from sputum.

Type 2: Friedländer B 5055, B 4209, B 7380 and 4631. All isolated from sputum.

B. *lactis aerogenes* 243 and 418.

No. 1360 freshly isolated from urine.

Type 3: Friedländer C 5056, C 10273, and X 4211.

No. 915, freshly isolated from sputum.

Rhinoscleroma C 5046, C 5047, C 5048, 1936 and 1383.

Type 4: Ozaena D 5050.

Type 6: Ozaena F 5052 and ozaena Diagnosis Department.

Type 7: B. *lactis aerogenes* 4140.

Types 8—14: All freshly isolated from urine (see Table 10).

From these results it will be noticed that within the *Klebsiella* group — just as in other groups — there is a small number of very common types, whereas many types are infrequently found. Investigation of a considerably larger number of strains will reveal many new but relatively rare types. As in other groups, the type distribution will be subject to variations due to differences in time and

location. Judging from the studies carried out so far, in the *Klebsiella* group we chiefly have to deal with capsular types 1, 2, 3, 8, 9, and 10, of which types 8, 9, and 10 are found mostly in urine.

The Diagnostic Antigenic Schema for the Klebsiella Group.

The establishment of a special antigenic schema for the *Klebsiella* group, independent of the *Escherichia* antigenic schema, was made for practical reasons. In the presence of *Klebsiella* strains it is more simple to have the sera required for the diagnosis entered in a special schema rather than looking for them in a large complicated *Escherichia* schema. Furthermore, as no H antigen is found in the *Klebsiella* group this schema comprises only two headings, O and K antigens. By combination of O antigens with K antigens, which in the *Klebsiella* group — in contrast to the *Escherichia* group — all are thermostabile capsular antigens, it is possible to set up a diagnostic antigenic schema as shown in Table 11. With a view to the use of this schema in practice, where a detailed determination of the O antigen is impracticable, the O partial antigens are omitted in Table 12.

Table 11.
Diagnostic Klebsiella Antigenic Schema (extended).

O groups	Antigens		Strains
	O	K	
1	1	1	A 4208, A 5054, 380
	1	2	B 5055, 243, 418
	1	3	C 5056, C 10273, 915
	1	7	Aer. 4140
	1	8	No. 1015 and others
	1	10	No. 919 and others
	1	12	No. 313
2 A	2a	2	B 4209
	2a, 2b	2	B 7380, 4631, 1360
	2a	3	Rh. 5046 and others
	2a	8	No. 1126
2 B	(2a), 2c, 2d	4	Oz. D 5050
	2a, 2c	5?	Oz. E 5051
	2a, 2c	6	Oz. F 5052
3	3	11	No. 390, 674
.	.	9	No. 56 and others
	.	13	No. 1470, 1995
	.	14	No. 1193

Key: Aer. = Aerogenes,
Rh. = Rhinoscleroma,
Oz. = Ozaena.

Table 12.
Diagnostic *Klebsiella* Antigenic Schema (simplified).

O group	Capsule type	Earlier designation
1	1	A
	2	B
	3	C
	7	.
	8	.
	10	.
	12	.
2	2	B
	3	C
	4	D
	6	F
	8	.
3	11	.
.	9	.
	13	.
	14	.

From Table 11 and 12 it will be noticed that capsular antigen 1 hitherto has been demonstrated only in O group 1. In contrast hereto, capsular antigen 2 occurs both in O group 1 and in O group 2. Type 1:2 includes strains Friedländer B 5055 and »Aerogenes« 418 and 243, while Friedländer strains B 4209, B 7380, and 4631, as well as strain 1360 (isolated from urine), belong to type 2:2. Strain B 4209 possesses O antigen 2 a, while the remaining 3 strains possess O antigen 2 a, 2 b. Correspondingly, capsular antigen 3 occurs in both O group 1 and 2. Strains Friedländer C 5056, C 10273, and 915 have formula 1:3, while all the rhinoscleroma bacteria have formula 2 a:3. These 2 types, 1:3 and 2 a:3, differ also culturally, as is evident from Table 2.

Also capsular antigen 8, which is most frequently found, occurs in both O groups, though more often in O group 1, as so far only one strain (1126) with formula 2 a:8 has been found.

All strains of capsular type 9 occurred in the R form, which thus appears to be characteristic of the type.

All the strains of capsular type 10 that contained any O antigen belong to O group 1.

If it is desired to continue the attempt to simplify the diagnostic schema, we may leave out the O determination entirely, making merely a diagnosis of the capsular types. This is recommendable in practice, as the isolation of acapsular forms required for determination of O antigen, or the production of sera with capsular forms, take a good deal

of time and, in the presence of R forms, will not lead to the desired result. In this case, then, the diagnosis should be given as *Klebsiella* type 1, type 2, etc. If an O determination is carried through, the types may be designated by their antigenic formula as: *Klebsiella* 1:1, 1:3, 2 a:2, 2 a:3, etc.

Also determination of the R antigens, which hitherto has not been carried through, will not come into consideration in the practical diagnosis, being merely of scientific interest.

Effect of Antibiotics on Klebsiella Cultures.

With a view to the frequent *Klebsiella* infections of the urinary tract and the therapy employed for such affections, studies have been carried out on the effect of penicillin, streptomycin, and sulfathiazole. More than 30 strains of the various types were tested in plate experiments (after the disc method). By means of a glass spatula a thin suspension of a culture in saline was spread on a 10 % horse blood agar plate, and 3 discs of filter paper (15 mm. in diameter) were placed on each plate. On one disc 1 drop of penicillin solution (200 I.U. per cc.) was placed; on another 1 drop of streptomycin solution (10,000 I.U. per cc.); and on the third 1 drop of 0.5 % sulfathiazole solution. The results were read after incubation at 37° for 20 hours. If no zone of inhibition was seen round the disc (i.e., less than 15 mm.) the culture was reckoned as resistant. If the zone of inhibition was between 16 and 24 mm. the sensitiveness was designated as 1, with a diameter of 25—34 mm. the sensitiveness was designated as 2, and with a diameter of 35 mm. or more as 3.

All *Klebsiella* cultures were resistant to penicillin and sensitive to streptomycin, the degree of sensitiveness lying between 2 and 3. The cultures differed in their behaviour to sulfathiazole, some being completely resistant, others showing a sensitivity between 1 and 3. A very old laboratory strain was resistant to sulfathiazole, so that the resistance cannot be due to treatment with the remedy. On the other hand, some experiments carried out by Erna Mørch-Lund (to be published later) showed that *Klebsiella* strains originally sensitive to streptomycin might become perfectly resistant in the course of treatment with this antibiotic.

From the above-mentioned experiments it is evident that streptomycin will be of primary therapeutic importance in *Klebsiella* infections. So in cases of pneumonia that do not promptly respond to treatment with penicillin the sputum should be examined bacteriologically in order to ascertain whether *Klebsiella* bacteria be present. For estimation of the resistance of *Klebsiella* cultures it makes no difference whether capsular or acapsular strains are employed or whether the test be carried out on plain agar or blood agar plates.

Mouse Virulence of Klebsiella Cultures.

In order to test the virulence of *Klebsiella* bacteria — especially the new capsular types isolated from urine — white mice (weighing 18—20 grams) were inoculated intraperitoneally with 0.2 cc. of 10-fold dilutions of a 20-hour broth culture. The mice were observed for 14 days, although death usually occurred within 48 hours unless the animal survived. Altogether 70 cultures were examined in this way.

Apart from some cultures which even in a dose of 0.2 cc. undiluted broth culture were unable to kill the mice, this dose proved lethal for a majority of the strains. In contrast hereto on dilution of the cultures the strains were divided into 2 groups. The strains of one group in culture dilutions even as high as 1:100.000 — 1:1 million proved lethal to the mice, whereas the strains of the other group in corresponding dilutions did not kill the animals. The first group included capsular types 1 and 2, even though not all the strains of this type were virulent (thus, for instance, strain 2:2, isolated from urine, was avirulent). The other, avirulent, group included all the strains isolated from urine, especially the frequently occurring capsular types 8, 9, and 10. As a rule these cultures are lethal to mice only as undiluted broth cultures, though sometimes they are lethal in dilution 1:10.

With regard to their pathogenicity, then, the *Klebsiella* strains fall in 2 large groups:

- I. Capsular types 1 and 2, which are virulent for mice; in man they mostly produce infections of the respiratory organs.
- II. Capsular types 8, 9, 10, and others, which are not virulent for mice, and in man they usually produce infections of the urinary tract.

The 2 groups do not differ biochemically, nor in their behaviour to antibiotics and sulfathiazole.

On the Nomenclature of the Klebsiella Bacteria.

In order to have a common designation for all the O groups and types of the *Klebsiella* bacteria it is recommended internationally to adopt the term »*Klebsiella group*« as already employed in the literature. As the classification of this group is based on the determination of serotypes, specific names for these types would be superfluous. We designate them after their antigenic formulae as *Klebsiella* 1:1, 1:2, 1:3, 2:2, 2:3, and so on.

Thus we put the *Klebsiella group* — which, in concordance with P. R. Edwards, is looked upon as one genus — on a par with the *Escherichia group*, which likewise is divided into serotypes without specific names.

This principle of classification, after which biochemically defined

groups are divided into serotypes, applies to the total group of Enterobacteriaceae (*Salmonella*, *Shigella*, *Arizona*, *Ballerup*, *Bethesda*, *Alkalescens*, *Proteus*, etc.), and it has proved most profitable for scientific and practical works.

Even though we fully realize that in nature — especially within the Enterobacteriaceae — there are no sharply defined groups, for practical reasons we still have to set up different groups by means of biochemical criteria. The results hitherto obtained through antigenic analysis show plainly that all these groups belong together so that in the future it will be possible to set up one antigenic schema covering all enteric bacteria.

Discussion.

If in keeping with Bergey's Manual we want to divide the tribe Eschericheae into biochemically defined genera, only 2 genera, »*Escherichia*« and »*Klebsiella*« should be set up, not 3 (*Escherichia*, *Aerobacter*, and *Klebsiella*). As belonging to the *Klebsiella* group we reckon a considerable number of Gram-negative capsular bacteria hitherto designated as *Friedländer*, *rhinoscleroma*, *ozaena*, *aerogenes*, and *aerobacter* bacteria. Owing to the rules of nomenclature, it is not permissible to designate this group as »*aerobacter* group« because the term »*Klebsiella*« has priority.

The *Klebsiella* group is defined as non-motile, Gram-negative, non-sporing rods that do not form indole and which ferment adonitol, inositol and other carbohydrates or alcohols, often breaking down urea and frequently giving a positive Voges-Proskauer reaction and negative methyl-red reaction. As a rule these bacteria grow on Simmons' citrate agar; normally they possess a capsule, and most of them form »mucus«.

In contrast hereto the *Escherichia* group (*coli* group) is made up mostly by motile, Gram-negative, non-sporing rods which usually form indole, ferment adonitol and inositol but rarely, and give a negative Voges-Proskauer reaction and positive methyl-red reaction. They do not decompose urea, and usually they do not grow on Simmons' citrate agar. Apart from the A forms and one L form, they possess no capsule, and as a rule they form no »mucus«.

As in all other groups of Enterobacteriaceae, there is no single criterion that is characteristic of the *Klebsiella* group exclusively nor of the *Escherichia* group alone. Still, a combination of some reactions will generally permit a decision as to the grouping of a given culture. There are cultures, however, that occupy an intermediate position, illustrating again that in nature no sharply defined group is to be found. Therefore, where it is not possible sharply to define the individual biochemical groups, the demarcation each from the other will be more or less arbitrary. There will always remain some strains

which cannot readily be entered in one of the groups. This applies not only to the *Escherichia* and the *Klebsiella* groups, but also to all other groups within *Enterobacteriaceae* — e. g., the *Salmonella* and *Arizona* groups. As already stated repeatedly, only the individual types can be defined sharply, not the various groups, among which transitions are to be found.

Serologically the *Klebsiella* strains examined so far belong to various O groups, the first and probably most frequent of which is closely related to the *Escherichia* O group 19, while the O antigen of the infrequent type 3:11 is identical with the O antigen of the *Escherichia* O group 9.

Here it will be appropriate explicitly to emphasize that so far the writer has investigated only the O and K antigens, not the R antigens.

Summary.

1. In contrast to the hitherto prevailing view — according to which the serology of the *Klebsiella* group is determined by carbohydrate-containing capsules and the R antigens consisting of protein — it has been demonstrated that *Klebsiella* bacteria contain also *O antigens*.

2. By means of the O antigens the *Klebsiella* group can be divided into various *O groups*.

3. The O'antigen in *Klebsiella* O group 1 is identical with *Escherichia* O antigen 19b, while the O antigen of *Klebsiella* O group 3 is identical with *Escherichia* O antigen 9.

4. By means of the capsular antigens it has been possible to divide the O groups into *types*, with the same capsules occurring in different groups.

5. A description is given of 8 new capsular antigens. The writer suggests that the capsular antigens should be designated by means of consecutive arabic numerals, as a large number of types must be considered.

6. *Klebsiella* capsule 10 is almost identical with *Escherichia* capsule 39.

7. Antigenic relationship is found between *Klebsiella* capsule 7 and *Escherichia* capsule 55, between *Klebsiella* capsule 8 and *Escherichia* capsule 34, and between *Klebsiella* capsule 11 and *Escherichia* capsule 37.

Thus *Klebsiella* type 3:11 has the same O antigen as *Escherichia* type 9:37, while capsules 11 and 37 have partial antigens in common.

8. By means of cultural methods the *Klebsiella* group can be divided into various *biochemical types*.

9. *Klebsiella* strains are non-hemolytic and as a rule do not cause hemagglutination.

10. Notwithstanding the close relationship between *Escherichia* and *Klebsiella* cultures, there still are such distinct biochemical dif-

ferences between them that they may be considered as constituting two special groups.

11. Attention is drawn to the pathogenic significance of the *Klebsiella* group, especially in *urinary infections* among which 3 new capsular types — 8, 9, and 10 — have been demonstrated in about 90 % of the cases.

12. Capsules types 1 and 2 are generally virulent for mice; in man they give rise mostly to *respiratory infections*.

13. Capsular types 8, 9, 10 and others are non-virulent for mice; in man they usually produce *urinary infections*.

14. As to the nomenclature of the *Klebsiella* group, the writer suggests that the term »*Klebsiella*« be accepted internationally and that the individual types be designated by their antigenic formulae (1:1, 1:2, 1:3, etc.), that is, leaving out all specific terms.

15. *Klebsiella* cultures are resistant to *penicillin* but sensitive to *streptomycin*. The individual strains vary in their behaviour towards *sulfathiazole*, but most of them are resistant.

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THE ETIOLOGY OF SEALER'S FINGER (BLUBBER FINGER, SPECK FINGER)

By Th. Thjøtta and Johs. Krillingen.

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The sealers of the northern and southern oceans are very often afflicted with an infection of the fingers called sealer's finger or blubber finger (Norwegian speck finger). This disease is only slightly known by text-book authors, but is very well known to practitioners residing in the northern cities of Norway who have to treat the sealers when they come home after their periods of seal hunting and present their swollen fingers for treatment.

It is the opinion of the sealers themselves that this disease is caused by some infection contracted through the handling of seals and especially through the skinning of the animals, when the hands of the skinner come into close contact with the carcass of the animal and some infective matter is rubbed into cuts and abrasions on the fingers. Experience justifies this view, but hitherto no microbe of any kind has been made responsible for the infection, but then it is true, only very slight interest has been taken in the elucidation of the problem. The reason for this fact is obvious. The infection is incurred far from civilization, where no physician is present, let alone an institute that might go into the problem. And when the patients reach home only the remains of the acute disease present themselves, and furthermore, if a finger of that kind is exarticulated it has to be sent a long way to reach a laboratory where the examination can be taken up.

Few papers on sealer's finger are to be found in the literature. The most exhaustive is that presented by *Mathiesen, Häupl* and *Thjøtta* in 1935. These authors have given an account of the disease, with regard to its clinical aspects, roentgenology, prognosis and treatment (1). A careful examination of all the material at hand in 1935 showed a number of disseminated infiltrations in the loose subepithelial connective tissue and especially in the fatty marrow of the finger bones. The articulations were often destroyed by infiltrations that had loosened the cartilage and partly resorbed it. This is the reason why

a finger affected by this disease very often is stiff after recovery. In this material no microbe was found that could be held responsible. The reason for this negative result was mainly thought to due to the poor condition of the material for a bacteriological examination. Almost all the fingers had been inflamed for a long time, and they were all dead material, where many saprophytes had invaded the tissues.

Since 1935 we have been interested in this disease. But only recently have we had an opportunity to take up this study again. This time we have had fresh material at our disposal, and we have obtained results that will be published — in spite of the fact that our material consists of only three cases — in the hope that other investigators may be induced to take up the problem.

Case I.

This was a typical case of »speck finger« with an infection of the third left finger that had lasted for 6 weeks. On the 29th of May a surgical incision was made and inflammatory tissue sent us for examination. On microscopical examination gram-positive cocci with the appearance of micrococci were found.

The material was inoculated into the following media:

Chocolate agar, blood agar, plain agar, semisubtle brain-heart extract agar, 1 per cent glucose broth. The incubation was carried out at 25, 30 and 37° C. aerobically and anaerobically and in a moist atmosphere.

After incubation for 48 hours all cultures yielded a growth of *Staphylococcus albus*. Besides these colonies, which were taken to be saprophytes, a few small, reddish-yellow colonies were seen. The latter colonies grew only in the aerobic cultures, but in cultures incubated at all the temperatures mentioned.

The reddish-yellow colonies were separated from the white staphylococci through several generations. The characteristics of the microbe thus obtained is as follows:

It is a gram-positive coccus, obviously a *Micrococcus*. The colonies are round, domed and smooth. The colors vary from dark yellow to red brick or rose, and the consistency is butyrous.

The optimal growth temperature was 30° C. The best growth occurred on chocolate agar plates, where colonies were visible after 48 hours' incubation and where they grew up to a size of 3—4 mm. after incubation for 8—14 days. The growth on common blood agar plates was slower and distinctly poorer. After several subcultures, growth was also obtained on plain agar, but it was slow and poor.

On gelatine no growth occurred, and on brain-heart extract agar in semisubtle condition very slight growth was seen on the surface only. In glucose broth a slight growth was seen after 8—14 days' incubation. Abundant growth and pigment production was found in litmus milk after 8 days' incubation.

No acid production was found in glucose-maltose and saccharose ascitic agar. The growth was slow and scanty.

Case II.

This case was an exarticulated finger with the symptoms of speck finger, but being an old case it was not very suitable for examination.

Cultivation was performed as in case I. Very abundant growth of white staphylococci and diptheroids was obtained. Among these colonies a single

yellow-red colony was found but isolation of this colony in pure culture proved impossible.

Case III.

This case was a fresh one. The material consisted of purulent tissue excochleated by Dr. A. F. Mathiesen*) in Aalesund, who sent us the specimen on the surface of chocolate agar in screw stopper flasks, with which we had provided him. On this medium we found a growth of white staphylococci and some reddish-yellow colonies that were subcultivated in pure form. These colonies and the microbe forming them were in every respect analogous to those described in Case I.

Thus, from two cases of speck finger we have elicited two strains of a microbe obviously with the same morphological and biochemical properties, and the question arise as to what microbe this may be and whether it has anything to do with the etiology of speck finger.

Taking the last question first, we have no convincing evidence that this microbe is the cause of speck finger. Repeated injections of the microbe into guinea-pigs and rabbits did not cause any disease in the animals, and complement-fixation and agglutinin experiments with blood from Case I did not give any positive reactions. Thus, it is exclusively on circumstantial evidence that we venture to call attention to this microbe. The microbe is obviously very seldom encountered. And this rare microbe has been cultivated from two cases of the same disease and demonstrated in a third. We are not surprised to find *Staphylococcus albus* as a saprophyte in material from the finger lesions. But it is of greater significance that we find a very rare microbe, that never seems to occur in ordinary cultures in material from a pathological lesion. And it means even more when two strains from different patients are shown to be identical morphologically, culturally, and serologically.

Serological examinations of the two strains were carried out in sera from rabbits inoculated with the two strains, partly killed and partly living emulsions. With these sera agglutination and complement-fixation experiments were made. The results were as follows:

			Serum I	Serum II
Agglutination:	Strain I	Titre	1:640	1:640
	Strain II	»	1:320	1:160
Complementfixation:	Strain I	»	0.0001 ml.	
	Strain II	»	0.0001 ml.	

*) We take this opportunity to express our gratitude to Dr. Mathiesen for his great help in providing us with material for this work. Dr. Mathiesen has always taken great interest in this disease both clinically and with regard to its treatment.

Both tests show that the two strains of our microbe are serologically identical.

We may thus conclude that we have cultivated two strains of a *Micrococcus* of a peculiar and rare species from our two cases of speck finger. This microbe produces a yellowish-red pigment and grows slowly and with difficulty on our media and does not ferment sugars nor liquefy gelatine. All these characteristics necessarily exclude this microbe from the known species of the large genus of *Micrococcus* ordinarily met with.

In our endeavor to classify this organism one of us (Thjötta) remembered to have at one time been working with a microbe very much like the one in question. This microbe was also a pigment producer and a gram-positive coccus with very slow growth, and, what was still more significant, it was cultivated from salt water fish. This was done as a study of the so-called red mite seen in dried and salted cod. This *Micrococcus* was shown to be a halophil microbe which could grow in solutions of sodium chloride up to 20 % and even more. We felt that it might be worth while to examine our strains as to their tolerance of sodium chloride, and very soon it was found that we really had to deal with a halophil microbe, a fact that seemed to give more hope of our microbe being a salt water microbe.

The experiments with salt solutions showed the following results:

Growth in 2½ per cent agar with various amounts of NaCl.			
NaCl per cent	Strain I	Strain II	
0	±	±	
1	+	+	
2	+	+	
3	++	++	
4	++	++	
5	++	++	
7	+	+	
10	±	±	

The optimal salt concentration is 5 %, and accordingly somewhat lower than in our red mite *Micrococcus*. But this is quite natural, since the *Micrococcus* from the fingers had lived for many generations in human tissue (if one may suppose so), while the red mite strain was cultivated directly from a very salt cod.

There is every reason to believe that our two strains were really salt water microbes, and if this is so, the evidence of their being the cause of speck finger is strengthened.

What may be the relation of this *Micrococcus* to the seal from

which the infection of the sealers is thought to come? The aforementioned study by Mathiesen, Häupl and Thjötta showed that the seal itself was affected by many lesions which upon histological examination showed very much the same picture. Häupl, who has a large experience in that kind of studies, felt that the lesions in man and in the seal must be due to the same insult. The seal obviously had obtained its lesions from rubbing against the ice or perhaps from battles with fellow seals. The open lesions in the skin may then well be infected with some microbe from the sea water and from the seal transmitted to the sealer during the skinning.

Conclusions.

1. From two cases of typical »speck finger« we have cultivated two strains of a microbe that has been identified as a pigment-producing strain of a *Micrococcus*, showing very slow growth on ordinary media. The microbe is moderately halophilic and has no fermentative powers against carbohydrates.

2. The isolated *Micrococcus* was only a moderately good antigen in rabbits. But after several injections the animals responded and yielded a serum that gave antibody reactions as well against the homologous as against the heterologous strain.

3. Although we cannot claim that this microbe is the cause of speck finger we feel that it may be a working hypothesis and we recommend other investigators to examine their cultures for this microbe when opportunity is at hand.

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SATELLITISM IN A CORYNEBACTERIUM DUE TO AN UNUSUAL GROWTH FACTOR

By *Emil Steen*.

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Hemophilus influenzae is known to grow as a satellite around other bacterial colonies on blood agar. A gram-positive rod which shows a similar satellite phenomenon, has been described by *Svendsen*, *Amlie* and *Dick Henriksen* (1) from this institute. They suggested the name *Corynebacterium hemophilum* for this rod because it required the growth factors X and V.

While examining a blood agar culture we found another *Corynebacterium* which showed a marked satellite phenomenon, growing around *staphylococcus* colonies. The culture originated from the nasal secretion of a patient suffering from acute iridocyclitis. The colonies largely resembled those of *Hemophilus influenzae* though they were more opaque and pearly grey. Further the satellite phenomenon was due to other growth factors than X and V.

Properties of the strain.

The *morphology* of the rod varied according to the size of the colony. The larger colonies near a *staphylococcus* colony consisted of non-sporing, straight, non-motile, gram-positive rods measuring about 1 by 3—4 μ . The rods showed clublike swellings of both ends which contained metachromatic, acid-fast granula. They showed a tendency to angular or parallel palisade arrangement, never forming chains. The smaller colonies consisted of coccobacilli which measured about 1 by 1.5 μ .

Meat Infusion Broth: Inoculation from a large colony yielded, after 24 hours, a clear medium, with a scanty, coarsely granular deposit, containing typical rods. After 2—3 days the broth became turbid and the deposit tenacious and homogenous. Microscopic examination mainly revealed coccobacilli. When the inoculation was made from a small colony, the broth became turbid with coccobacilli within 24 hours.

After 30 subcultures in meat infusion broth, the microbe still presented the typical satellite phenomenon on blood agar.

Colony: The colonies were on the whole pinpoint-sized circular, slightly elevated, smooth, pearly grey, and semi-transparent. Close to the staphylococcus colonies they were somewhat larger, measuring up to 1—2 mm. in diameter.

Fermentation reactions: Various sugars, alcohols, and glucosides were added to peptone water. Acid, but no gas was produced from levulose and saccharose only, but not from glucose.

The Satellite Factor.

Grassberger (2) as early as 1898, observed the well-known phenomenon that *Hemophilus influenzae* grows in large colonies near staphylococcus colonies on blood agar. Neisser (3) suggested the name »Ammenbacillen« for bacteria which thus favour growth. The term »Satellitisme culturel« was first introduced in 1919 by Meunier (4).

Theoretically, the satellite phenomenon may be produced in two ways:

1. An organism may produce substances which favour the growth of another organism.
2. An organism may exert a destructive or inhibiting effect on substances which inhibit the growth of another organism.

The production of substances favouring growth is the usual cause of the satellite phenomenon. Thjötta and Avery (5) in 1921, showed that *Hemophilus influenzae* requires the X and V factors. At present we know that the X factor is identical with hemin, and Lwoff and Lwoff (6) have shown that the V factor is identical with Hardner and Young's cozymase and Warburg and Christian's coenzymes (di- or triphosphorpyridine nucleotide).

Hemin is probably the growth factor, the chemical structure of which was first determined. Later on a number of growth factors were detected and partly identified. Koser and Saunders (7), Peterson and Peterson (8), and Porter (9) have given excellent surveys on this subject. Different microbes vary considerably in their requirements for growth factors and ability to synthesize their own growth factors.

Müller and Schopfer (10) in their publication dealing with thiamin, mention how the presence of one micro-organism may favour the growth of another. *Mucor rammanianus*, a mold fungus is incapable of synthesizing one component of the thiamin molecule. *Rhodotorula rubra*, a yeast fungus, needs only the other thiamin component. These fungi are thus not capable of growing separately in simple synthetic media without thiamin. Together, however, they grow readily without thiamin being added, as one fungus produces the part of the molecule which is required by the other.

Another example is described by Kögl and Fries (11). The two

fungi, *Polyporus adustus* and *Nematospora gossypii*, will not grow separately on simple media. However, when inoculated together they develop, for the simple reason that *Polyporus* requires thiamin which *Nematospora* is capable of producing. On the other hand, *Nematospora* requires biotin which is produced by *Polyporus*.

The satellite phenomenon on blood agar has, as previously mentioned, been described for a *Corynebacterium*, which requires the X and V factors. These factors, however, exert no influence upon the strain which we have isolated. Nor did we see any effect of potato or turnip extract, thiamin, nicotinic acid, pantothenic acid, pyridoxine, para-aminobenzoic acid, and riboflavin.

In order to determine which factors might be responsible for the increased size of colonies growing in close proximity to colonies of staphylococci, some experiments were carried out, with a view to the following:

1. Which bacterial species have the growth-promoting effect?
2. On what kind of media will the satellite phenomenon occur?
3. What are the properties of the growth factor?

Ad 1.: The *Corynebacterium* was streaked on blood agar plates in parallel streaks, whereas the microbes to be tested were streaked at right angles to the *Corynebacterium* in widely separated streaks. A total of 46 different strains were tested, but a growth-promoting effect was obtained by *Serratia marcescens* and by 6 out of 8 strains of *Staphylococcus aureus* only.

Ad 2.: The *Corynebacterium* was inoculated together with yellow staphylococci on various media. The technique just described was used. The results are shown in table 1.

Table 1.

Medium	Satellite Phenomenon
Ordinary agar	—
Loeffler's ox serum	+
Ascites agar	+
Agar with 5 % washed blood* cells	—
» » 5 % whole blood*	+
» » 5 % serum*	+

* Blood from man, sheep, rabbit, and horse gave identical results.

It will be seen from the table that the satellite phenomenon occurs on agar containing serum, but not on agar containing washed blood cells only. Whether human blood or blood from sheep, rabbit, or horse is used, the results are the same.

Ad 3.: In order to investigate the growth factor the following strains were grown in fluid media: *Serratia marcescens*, *Staphylococcus aureus* B. 382, a staphylococcus with marked growth-promoting effect, and *Staphylococcus aureus* S. 174, a strain which showed no growth-promoting effect on blood agar plates.

The following media were used: Ordinary meat infusion broth, broth to which was added 10 % serum, 10 % washed blood cells, or 10 % whole blood.

The cultures were incubated under aerobic conditions at 37° C. for two days. They were then filtered through a Berkefeld filter and adjusted to a pH of 7.8. Blood agar plates were inoculated with the *Corynebacterium*. Cylindrical holes were punched in the blood agar plate and filled with the various filtrates. The plates were incubated in a moist atmosphere in order to prevent evaporation, and examined after two days. The results are shown in table 2.

Table 2.
The Growth Factor in Filtrates from Fluid Cultures.

Medium	Staph. aur. B. 382	<i>Serratia</i> <i>marcesc.</i>	Staph. aur. S. 174
Meat infusion broth	—	—	—
M. i. broth + 10 % serum	+	+	—
M. i. broth + 10 % blood cells	—	—	—
M. i. broth + 10 % whole blood	+	+	—

+ indicates satellite phenomenon around the hole in the blood agar.

— indicates no satellite phenomenon around the hole in the blood agar.

Meat infusion broth containing 10 % hemolysed human blood was used as control.

The table shows that the growth factor is filterable and is formed when *Serratia marcescens*, or *Staphylococcus aureus* B. 382 grows in meat infusion broth containing serum.

In the following experiments a filtrate from a serum broth culture of B. 382 was used. The filtrate was tested for thermoresistance and it was found that the growth-promoting factor resisted a temperature of 65° C. for 20 minutes, but was destroyed when heated to 75° C. for 20 minutes. The growth factor was not dialysable through a cellophane membrane.

The globulins and albumins contained in the filtrate were precipitated by ammonium sulphate at 50 % and 100 % saturation respectively. The various fractions were redissolved in isotonic saline and the ammonium sulphate removed by dialysis against 0.85 % NaCl. The pH was adjusted to 7.8 and the samples tested for sterility. More-

over, trypsin was added to the original filtrate and the mixture incubated for two days at 37° C. These solutions were then all tested for growth-promoting effect. The results are shown in table 3.

Table 3.

Solution	Satellite phenomenon
Original filtrate	+
Trypsin-treated filtrate	—
Globulin fraction	—
Albumin fraction	+
Filtrate less the globulin fraction	+
Filtrate less the albumin and globulin fraction	—

The table shows that the growth-promoting factor is precipitated by ammonium sulphate, together with the albumins, and that it is destroyed by trypsin.

Comments.

The gram-positive rod described probably belongs to the genus *Corynebacterium*. The pleomorphism, i. e., the typical rods and coccobacilli, and the different growth in meat infusion broth, are the same as that of *Corynebacterium enzymicum*. On the other hand, the cultural features resemble more closely those of *Corynebacterium xerose*. The most characteristic feature, however, is the satellite phenomenon which occurs when the microbe grows near a staphylococcus colony on blood agar. This was a constant feature for more than two months, the period during which the microbe was cultured in the laboratory. *Corynebacterium hemophilum* shows the same satellite phenomenon on blood agar but requires the factors X and V for its growth. These factors had no influence upon the strain we have isolated. Consequently this strain cannot be identical with any of the previously described species of *Corynebacterium*.

Mueller (12) has shown that some corynebacteria, particularly *C. diptheriae*, require a number of growth factors. The best known are nicotinic acid, beta-alanine, and pimelic acid. The two latter may be replaced by biotin and pantothenic acid. The growth factor which we have investigated, however, is of the nature of a protein, or at least in its active form, linked to a protein. It is therefore obviously not identical with the previously known growth factors required by different strains of *Corynebacterium*. The following reasons strongly suggest that the factor is a protein: It is only produced in serum-containing

media. It is filterable but not dialysable. Further, it is thermolabile and is destroyed at temperatures which cause precipitation of proteins. The most important point, however, is the fact that it can be precipitated by ammonium sulphate and that it is destroyed by trypsin.

Summary.

A *Corynebacterium*, which grows as a satellite around staphylococcus colonies on blood agar, is described. The satellite phenomenon is due to a filterable growth factor which is produced when staphylococci grow in broth containing serum. The growth factor is a protein, probably an albumin, and is not identical with the X or V or other known factors promoting the growth of *Corynebacterium*.

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A CONTRIBUTION TO THE QUESTION OF THE POSSIBLE PHAGOCYTOTIC ABILITY OF THE MEGA- KARYOCYTES

By *Georg-Fredrik Saltzman*.

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Great difficulties have been found to be attached to the study of the megakaryocytes with regard to manifestations of life and physiologic functions. It is probably impossible, at present, to state definitely whether any investigator has, on the whole, seen a living megakaryocyte. Those few hematologists who have studied these cells in bone-marrow cultures and in native preparations have not been able to demonstrate any definite phenomena of life. Whether this failure is due to the fact that the megakaryocytes do not survive the transference from their natural environment according to the general methods, or perhaps to the fact that the giant cells of the bone marrow even under physiologic conditions are unable to show the signs of life which are typical of other blood and bone-marrow cells, remains an open question.

Certain observations, however, have been considered to elucidate, indirectly, the functions of the megakaryocytes in the human organism. In a previous work (11) I have discussed the question of the part played by the megakaryocytes in the platelet formation. The possible phagocytic ability of these cells is another question of great interest to many investigators.

According to the general opinion the term phagocytosis includes all those processes by which a cell, actively, takes up foreign substances from the surroundings. In the literature the ability of the megakaryocytes to ingest other cells has been the chief centre of interest. The present communication will also deal chiefly with this aspect of the problem, whereas, for instance, the ingestion of bacteria, which is important although it has practically never been studied so far as the megakaryocytes are concerned, will be left open to further consideration.

Preparations fixed in different ways are often found to contain

megakaryocytes which have, seemingly, in their cytoplasm taken up blood corpuscles of various kinds. Naturally enough, such pictures led to the earlier prevailing opinion that the megakaryocytes had the properties of phagocytes. Also *Wright* with his world-wide reputation as an investigator on megakaryocytes upheld this view. Already in 1911, however, *Maximow* maintained that the megakaryocytes were not capable of phagocytic action. Later he drew attention to the fact that the majority of so-called phagocytosed elements were leucocytes, and proposed that these mobile corpuscles would, actively, approach the megakaryocytes. As *Maximow* points out, it is very difficult, if not impossible, in our usual preparations and with the ordinary optics to distinguish between actually phagocytosed cells and those which are situated on or underneath the megakaryocyte or in deep folds in its cytoplasm.

Many authors have also later followed this line of thought. A complete survey of the literature on this subject would lead too far. I will mention in the first place *Introzzi* and *Wuyts* who in their reviews give all the essential references. A few works which in particular illustrate the attitude of later investigators to the problem may be mentioned briefly here.

Firket, partly in co-operation with *Bouille*, has made a close study of the phagocytic ability of the megakaryocytes. These investigators found that pictures which would show an unmistakable phagocytosis of red or white corpuscles were extremely rare; they questioned, indeed, their existence on the whole. They observed also that the megakaryocytes did not stain with ordinary vital stains, which in their opinion would disprove the phagocytic ability of these cells. According to these authors, on increased dissolution of erythrocytes, as the result of pathologic conditions or produced experimentally, iron could not be demonstrated in the cytoplasm of the megakaryocytes. On the basis of these observations the authors considered themselves justified in renouncing the opinion that the megakaryocytes would possess phagocytic ability.

According to *Kingsley* the pictures which had earlier been considered to show megakaryocytes with phagocytosed cells, were incidental findings conditioned by the fact that the ordinary microscope allows only two-dimensional vision. Thus, these so-called phagocytosed cells, according to *Kingsley*, would be situated incidentally in folds of the megakaryocyte cytoplasm. This view was later supported by *Potter & Ward* and *Leitner*.

Torrioli on examining megakaryocytes in native preparations from bone marrow, partly even in dark-ground illumination, has found no evidence of phagocytic action. *Undritz & Rothlin*, quite recently, have refuted the idea of phagocytosis, emphasizing their opinion that the so-called phagocytosed cells are incidentally imposed.

Fieschi and Astaldi, on examining megakaryocytes in bone-marrow cultures, were also unable to observe evidence of phagocytosis.

Domenighini & Braghin, on the other hand, believed, not so long ago, that they were able to demonstrate definite phagocytosis in 4.68 per cent of the 15000 megakaryocytes examined by them. In their opinion the possibilities for the process had been proved, but they did not regard the phagocytosis as an integral phenomenon of life in the megakaryocytes.

It would perhaps be of some interest to report here some observations made in native preparations on the heating table in connection with my investigations on the origin of platelets (11). When the preparations were examined microscopically, immediately after having been produced, the great majority of the megakaryocytes appeared completely isolated from other bone-marrow elements. In those few instances where leukocytes were seen close to the megakaryocytes, the possibility of chance could not, at any rate, be ruled out. Very soon, however, neutrophilic and eosinophilic granulocytes began to collect about the megakaryocytes with the speed which is typical of the intact leukocytes in motion. During this process the leukocytes were the active cells. The megakaryocytes remained completely immobile without showing the slightest changes in shape or any definite signs of life whatsoever. It should be mentioned, however, that some of the leukocytes in the vicinity did not approach the megakaryocytes. Only the neutrophilic and the eosinophilic granulocytes, thus, the most active leukocytes, were seen to approach the giant cells in the above-described manner. It should perhaps be added that this migration of leukocytes was never observed in preparations where the white corpuscles showed no signs of life.

Shortly, the white corpuscles began, seemingly, to make their way into the cytoplasm of the megakaryocytes. At this stage the observations were facilitated by the use of the microscope with a dark-ground system. By raising and lowering the objective it was easy to observe that the leukocytes were situated either above or underneath the megakaryocyte, usually above. I have never been able to detect such a leukocyte surrounded by megakaryocyte cytoplasm both above and underneath. The difference in coarseness of the pale granules in the cytoplasm of these two cells is sufficiently evident to rule out the possibility of errors.

Just in these experiments I have found it most easy to compare the coarser and equal-sized pale drops of the neutrophilic granulocytes with the considerably smaller and unequal-sized pale drops of the megakaryocytes. This comparison has been of particular interest to me in connection with my studies on the origin of platelets (11). I mention intentionally only the neutrophilic granulocytes. The pale

drops in the eosinophile blood cells are so large that even the most inexperienced observer without difficulty will be able to distinguish them from the pale drops of the megakaryocytes.

During this process the megakaryocytes remained completely passive. It was not unusual that a white corpuscle which for a while had been projected in this manner towards a megakaryocyte, would withdraw again, in some instances to return, in others for the rest of the observation period. Most of these leukocytes, however, remained in intimate contact with the megakaryocyte, until they ceased to show any signs of life.

This process was not observed only sporadically. On the contrary, in the great majority of preparations neutrophile and eosinophile granulocytes gathered in this manner about all the megakaryocytes. In a few instances giant cells without leukocyte satellites were seen, but this should undoubtedly be regarded as fairly rare. The number of white corpuscles gathered about the different megakaryocytes varied. It was not at all unusual to see some ten leukocytes about a giant cell.

In the great majority of instances the leukocytes were not able to injure or otherwise change the megakaryocyte in any noteworthy degree. They would dent the periphery of the megakaryocyte on approaching the cell, but these minor changes were seldom permanent, the natural shape being restored spontaneously. As the leukocyte on its way over or underneath the megakaryocyte appeared to require the bigger part of the space between the cover glass and the object glass, it had to push aside the necessary amount of the granulated cytoplasm of the giant cell. Therefore, it usually left behind an optically more or less void groove in the cytoplasm of the megakaryocyte. This groove was usually closed very rapidly and some minutes after the leukocyte had passed there were no traces whatsoever of its course. In most instances the leukocytes evaded the nucleus of the megakaryocyte. The nucleus appeared to be able to resist more effectively the efforts of the leukocyte to change its shape.

One instance which I have mentioned previously in a different context (11) seems to deserve to be mentioned again. A megakaryocyte with unusually sparse cytoplasm and without observable manifestations of life, was, in the manner described in the foregoing, surrounded by a few neutrophile granulocytes. In the usual manner these white corpuscles began to make their way into folds of the megakaryocyte cytoplasm. Contrary to what had been the case in all my other observations, one piece of cytoplasm after the other was split off from the megakaryocyte. Most of these fragments remained lying in the immediate vicinity of the megakaryocyte. In one instance, however, one of the white corpuscles disappeared from the field of vision with two fragments of cytoplasm in tow. It withdrew so far that I dared not follow the fate of this remarkable »train« at the risk of losing sight.

of the megakaryocyte. Finally, the nucleus was stripped of all its cytoplasm by the remaining leukocytes.

This isolated observation does not seem to have anything in common with the manner in which the megakaryocytes usually react to the attacks of the white corpuscles. It would be obvious to presume that the cell had been injured in some unusual way. This appeared highly probable, particularly as neither the intact megakaryocyte nor the extruded fragments showed the slightest sign of typical blister formation or of accumulation of fibrin (11).

The observation is interesting, however, in that it shows that there are instances in which the resistance of the megakaryocyte is greatly reduced, while, at the same time, permanently favourable conditions for the manifestations of life in the leukocytes remain. I have again the platelet formation in mind, although, naturally, I dare not draw any conclusions whatsoever from this isolated case.

Which, then, are the forces that drive the leukocyte towards the megakaryocytes in the manner described here? Well, the answer to this question cannot be given on the basis of these experiments. As a result of my observations I find it difficult to accept the idea of an altogether incidental contact, as proposed by *Undritz & Rothlin* and *Kingsley*, et al. A suitable means of studying this question closer, however, seems to be examination of native preparations in dark-ground illumination.

In my opinion the literature contains no conclusive evidence that the megakaryocytes would be capable of genuine phagocytosis. The observations described here have given still less evidence in this direction. Although my experiments may not be said to have been carried out under physiologic conditions, an account of the observations seems to be justified. The picture presented by many megakaryocytes after a fairly short period of observation, harmonizes completely with those pictures which have given rise to and been interpreted in favour of the theory of phagocytosis.

Similarly to the question of the origin of platelets, the problem concerning the possible phagocytic ability of the megakaryocytes will not be finally solved until bone-marrow preparations can be examined under wholly physiologic conditions. Insofar as the above-described modest observations have served to rectify earlier misconceptions, they have had a small mission to fulfil.

Summary.

After a review of the attitude of recent investigators to the question of the possible phagocytic ability of the megakaryocytes an account is given of studies of these cells in native preparations from bone marrow, principally with dark-ground illumination. These observations verify that the megakaryocytes do not possess phagocytic ability.

but that the pictures which have been interpreted in favour of this property, are conditioned by a tendency of the mobile white corpuscles to accumulate about the giant cells. The reason why the leukocytes are driven in this manner towards the megakaryocytes cannot be determined. The problem in its whole extent will apparently not be finally solved until it becomes possible to study bone-marrow cultures under wholly physiologic conditions.

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SERO-TYPES OF A COLLECTION OF MUCOID GRAM-NEGATIVE RODS

By *Sverre Dick Henriksen.*

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Considerable knowledge has been gained about the antigenic structure of the various members of the tribus *Escherichiae*.

In genus *Klebsiella* Julianelle (1926, 1930) established the existence of three distinct sero-types, A, B and C and a heterogeneous group, X, in a collection of strains associated with pneumonia and other severe infections. This work was extended by Goslings (1933, 1935, 1936), Goslings and Snijders (1936), Wielenga (1937) and ten Have (1943), who showed that all rhinoscleroma strains were serologically identical with a capsular antigen (C) which was serologically indistinguishable from that of Julianelle's type C, although differences might possibly be detected by means of very specific phage strains. Ozaena strains could be separated into the predominating sero-type D and the less frequent types E, F, AE and C. Each of the species *K. pneumoniae*, *K. rhinoscleromatis* and *K. Ozaenae* was shown to have a separate somatic antigen (or partial antigen).

In the genus *Escherichia* the existence of a large number of different capsular, somatic and flagellar antigens has been established by Kauffmann (1943, 1944), Kauffmann and Vahlne (1944), Knipschildt (1945), Vahlne (1945), Sjöstedt (1946) and others.

Aerobacter, finally, has been studied less extensively. It has been shown that different somatic and capsular antigens can be found in this genus. At the same time cross-reactions with members of the other genera have been demonstrated repeatedly, thus with the capsular antigens of *K. pneumoniae* type B (Edwards, 1929, Julianelle 1937, Goslings 1933 and Wielenga 1937), and with somatic antigens of *Klebsiella* (Goslings 1933, Wielenga 1937) or *Escherichia* (Vahlne 1945).

When the present study was started, the author was not aware of the very careful studies by Wielenga (1937) and ten Have (1943), but in spite of the fact that these works have clarified our knowledge of mucoid gram-negative rods very considerably, it was decided to continue the study, as it was felt that an examination of a different collection of strains might be of some value as a confirmation — or otherwise — of the results of the Dutch workers. Another point was also kept in mind, namely that the strains previously studied have mostly been collected from certain distinct diseases (pneumonia, ozaena or rhinoscleroma), whereas little is known as to the

nature of the mucoid strains that are frequently found in a variety of different diseases.

Material. The strains were isolated from routine material received in this laboratory in the course of a couple of years. Any strain that was sufficiently mucoid to be classified as, or mistaken for *Klebsiella*, was included, regardless of source. The majority of strains were isolated from mild infections or from diseases of non-infectious or questionable origin. Only one severe case of *Klebsiella* infection occurred in this period (a fatal case of liver abscess). Thus there might be reason to expect that the collection would include members of all the three genera of *Escherichiae*. The very common slightly mucoid type of *E. coli*, which may be found in feces or urine, was not included.

For comparison with these strains a number of type strains of *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* were made available through the courtesy of Drs. Marion C. Morris, New York City, professor E. P. Snijders and J. W. Wolff, Amsterdam and Martin Kristensen, Copenhagen. My thanks are due to these doctors.

Origin of the strains.

Ozaena, simple chronic rhinitis and sinusitis	13 strains
Ozaena tracheae	1 "
Asthma and chronic bronchitis (sputum)	16 "
Pneumonia, not caused by <i>Klebsiella</i> (sputum)	3 "
Liver abscess	1 "
Other diseases, not due to <i>Klebsiella</i> infection	2 "
Urinary infections	15 "
<i>Klebsiella</i> type strains	12 "

Some other mucoid strains were found not to belong in this group. A few of these strains have been described elsewhere (1918).

Methods.

Immune sera were produced by intravenous injections of rabbits with formalin-killed saline suspensions from agar slants. If satisfactory titers were not obtained, these injections were followed by a few of living antigens. Injections were usually given on 3 or 4 consecutive days of each week, for 3 weeks or more. Dosage varied according to the tolerance of the animals. Bleedings were made 6 days after the last injection, and the serum stored in the cold without preservative. Slide agglutination tests were carried out by suspending a small quantity of organisms from a lactose agar culture in a drop of saline and adding serum in proportions from 1:10 to 1:1 according to the titer of the serum. The use of concentrated serum did not lead to non-specific cross-reactions. Complement fixation tests were carried out with twofold dilutions of inactivated immune serum, antigen suspensions containing about 1000 million organisms per ml and two hemolytic units of complement, all in 0.2 ml volumes. Incubation at 4 C for 18–20 hours followed by addition of 0.4 ml 1.5 % sensitized sheep erythrocytes. The tests were read after 15' at 37 C and again after about 2 hours at room temperature. Positive reactions were found to be stable and reproducible within one tube above or below the actual titer.

Cultural and biochemical tests. All strains were examined as to type of colony, morphology of the organisms, motility, type of growth in broth, and for capsules (by an India ink method). The following differential media were used: fermentation tubes with lactose, mannitol, maltose, glucose and saccharose, peptone meat infusion broth for indol production, nitrate medium,

lead acetate agar, nutrient gelatin and glucose broth for methyl red test and the Voges-Proskauer reaction. Finally a number of strains were tested for growth in Koser's citrate medium.

Results.

The slide agglutination method was found to give entirely specific results, and no cross-reaction between the different sero-types or with unrelated mucoid organisms was ever noticed. A number of test tube agglutinations were set up for comparison, but did not yield any more information than the slide tests. Also the test tube titers varied a great deal according to the density or content of capsular substance of the individual antigens, and titers were invariably low (from 1:10 to 1:160, mostly 1:20 or 1:40). The impression was gained that all the required information could be obtained by qualitative tests and that determination of the titers gave no advantage (with a few exceptions to be dealt with later). Positive results of the slide method appeared immediately and were characterized by the formation of a voluminous, fibrous precipitate.

3 of the strains lost their capsules at an early stage, and consequently could only be tested in one or a few immune sera. These strains were motile and apparently belonged to *Escherichia*. All the other 60 strains were tested in sera against the following organisms: P 32 (Julianelle's type A), NY (type B), F 10 (type C), MA 752 (type D), S 57-0 (type AE), S 57-P (*K. paulum fermentans*(?), ten Have 1943) and MA 248 (mucoid *E. coli*). The results are shown in table no 1. It should be mentioned that the serum S 57-0 was very weak and only gave moderate reaction in concentrated form and no cross-reactions with *K. pneumoniae* type A. The strain apparently was a poor antigen as only one out of two rabbits produced any capsular antibodies at all.

Of the 8 strains reacting with serum P 32 (type A), 5 appeared to be typical type A strains, whereas 3, one of which was isolated in this laboratory, culturally and biochemically belonged to *K. ozaenae*, and also reacted in serum S 57-0. Thus they were obviously type AE strains.

Of the 4 strains which reacted in serum NY (type B) only the two type strains (NY and F 24) were typical strains of *K. pneumoniae*. The two others, which were isolated from the same case of urinary infection with an interval of several months, were classified as *Aerobacter*, both because their biochemical properties were those of *Aerobacter*, and because they did not absorb all type B antibody from the immune serum (table 2). This, then, is another instance of cross-reactions between *Aerobacter* strains and *K. pneumoniae* type B.

Of the 6 strains reacting with serum F 10 (type C), two were type strains (two cultures of Julianelle's F 10, obtained from different laboratories), two were typical strains of *K. rhinoscleromatis*, whereas two showed most of the biochemical reactions of *Aerobacter*. One of

them absorbed only a fraction of the type C antibodies, whereas the other seemed to absorb all type-specific antibodies (table no 2). Thus capsular substance, cross-reacting or identical, with the type C specific substance may be found in *Aerobacter* strains.

As many as 30 typical strains of *K. ozaenae* reacted with serum MA 752 (type D). This is in complete agreement with the findings of the Dutch authors (Goslings 1933, Wielenga 1937, ten Have 1943). It appears that *K. ozaenae* type D is by far the most frequent type of *Klebsiella* both in ozaena and in other mild infections or non-infectious diseases such as asthma, chronic bronchitis and simple chronic rhinitis. It should be emphasized that all our strains of *Klebsiella*, with one exception (a type A strain from a liver abscess) were found in the respiratory tract. No typical *Klebsiella* strain was isolated from the urinary tract nor from feces.

The fact that as many as 9 out of 15 strongly mucoid urinary strains reacted in the same serum, with a typical carbohydrate-anti-carbohydrate reaction, is surprising. This point will be taken up in the discussion. All these strains were typical strains of *E. coli* by their cultural and biochemical reactions.

Complement fixation tests. It was claimed in a previous communication (1948) that this reaction was well suited to the study of *Klebsiella* strains. Although this claim could be substantiated to some extent as far as *K. ozaenae* is concerned, the sera produced with *K. pneumoniae* were far less specific and did not show any separation between the different *Klebsiella* species (table no 3). It is found that serum MA 752 is fairly specific and shows only moderate cross-reactions with certain strains of *E. coli* and *Aerobacter*, none at all with the other *Klebsiella* species. The serum is species-specific, however, as it reacts to the same titer with types D and AE.

The immune sera against *K. pneumoniae* types A, B and C were quite non-specific, and gave about equally strong reactions with all types of *Klebsiella* and with *Escherichia* and *Aerobacter*. It appears that these sera contain some group specific antibody, common to all members of tribus *Escherichiae*. This is surprising since capsule-free strains of the different species show a high degree of species-specificity (*Klebsiella*) or O-group specificity (*Escherichia* and possibly *Aerobacter*) in the agglutinin test. Thus, the antigens brought to light by these tests must be different from those that determine species-specificity or O-group specificity respectively. In the immune sera against strains of *E. coli* (MA 248 and MB 700) the reactions with the homologous strain were somewhat more specific, probably due to the presence of O-antibodies of high titer, but group-specific antibodies of a similar nature as in the *K. pneumoniae* immune sera also appear to be present. Thus, although the results were disappointing as far as separation of the different species and types are concerned, they give other information of some interest.

Agglutination reactions with autoclaved antigens. In view of the fact that Vahlne (1945) got satisfactory results with the inagglutinable A-strains of *E. coli* by using autoclaved antigens, some tests were made in the same direction with *Klebsiella* antigens, although it was realized that success was unlikely (thus Mikulaszek prepared serologically reactive capsular substance from autoclaved cultures 1929). Some results are shown in table no 4.

Although most of the antigens became more easily agglutinable after autoclaving, they reacted practically only in the homologous immune sera, and only very slight signs of species-specific agglutination were found. The results may indicate that the capsular substance was reduced in quantity by the procedure (autoclaving at 120 C for 2 hours), but still present in sufficient amount to determine the reactivity of the organisms.

Cultural and biochemical properties. A study of the colony seems to be of some value, as it has been noted (Goslings 1933, ten Have 1943) that there are some slight differences between the different serotypes of *Klebsiella*. It appears that the nature of the capsular polysaccharide to a certain degree determines the shape and appearance of the colony. In our collection of strains the following different types of colonies were found.

I. Large, domed, semitranslucent colonies with only a moderate tendency to coalesce. In the resulting conglomerate colonies, the outlines of each constituent colony can generally be distinguished. The consistency is very viscous, somewhat stringy, and it is a little difficult, and takes some time, to prepare even saline suspensions. This type of colony was found in all strains of *K. pneumoniae* type A and in the strain of *K. ozaenae* type AE.

II. Large, comparatively flat, watery or syrupy colonies with a marked tendency to form amoeboid figures. The colonies were greyish, semitranslucent or nearly quite translucent with an only slightly viscous consistency. This colony was found in all strains of *K. ozaenae* type D, and in the two strains of *K. rhinoscleromatis* that were seen (it is possible that the latter were slightly more domed and of a slightly firmer consistency than *K. ozaenae* type D).

III. Raised to domed, greyish white, comparatively opaque colonies, somewhat smaller than the others, with a moderate tendency to coalesce and a soft creamy consistency. This colony was found in the two strains of *K. pneumoniae* type B and in two different cultures of type C (F10). One of the latter cultures however (from Amsterdam) was more viscous and larger than the other (from New York). The mucoid strains of *Aerobacter* also grew with this type of colony, but 3 of the 4 strains were more watery and more strongly mucoid than the others.

IV. The mucoid strains of *E. coli* that were included in the study grew with colonies that resembled those of type I, but as a rule they were considerably larger, strongly domed and usually semitranslucent or nearly translucent. Apart from the appearance of the colonies, the individual strains were different. The colonies of some of the strains had a consistency like a jelly, from which small pieces could be broken off without any sign of stringiness. Others were viscous to the same degree as type I, whereas two strains (isolated from the same patient with an interval of several

months) were extremely viscous and though, strongly adherent to the agar surface. It was very difficult to remove a portion of a colony with a loop. All colonies in this group were difficult to emulsify in saline, but finally formed even, very viscous suspensions. All these colonies gave a very strong precipitin type reaction with the same immune serum (MA 248).

It should be noted that the difference between the colonies was most marked in recently isolated strains, incubated at 37 C, whereas in older strains and in cultures incubated at room temperature the difference sometimes became less marked.

The study of the other cultural and biochemical reactions did not yield any new information, but confirmed the results of the Dutch workers. Only a summary of the results is presented.

Table no. 4.

Slide agglutination tests with 63 strains of encapsulated gram-negative rods.

Immune serum	Number of agglutinated strains	Origin of strains
P 32 (type A)	6 + 3*	Fatal liver abscess, rhinitis, sinusitis (nose), asthm. bronchitis, sinusitis (pus)*, type A strains (2), type AE strains (2)*.
NY (type B)	4	Urinary infection (2)**, type B strains (2).
F 10 (type C)	6	Urinary infection (2)**, type C strains (2), rhinoscleroma type strains (2).
MA 752 (type D)	31	Sputum (asthma, bronchitis, pleurisy, bronchopneumonia) (14). Nose (ozæna, rhinitis, sinusitis) (8), pus from sinus (2), empyema, eye, abscess, throat, trachea (ozæna), unknown, type D strain.
S 57-0 (type AE)	3	Sinus, AE type strains (2).
S 57-P (?)***	1	Sinus.
MA 2 48 (E. coli)	8	Urine (7), ulcerating sarcoma.
Not typed	4	Sputum, urine (2), F type strain.

*: cross-reacting type AE strains.

**: cross-reacting strains of *Aerobacter*.

***: possibly *Klebsiella paulum fermentans* (ten Have).

Table no. 2.
Cross-absorption tests with *Aerobacter* strains and *K. pneumoniae* types B and C.

Serum	Absorbed with	Agglutination with		
		A. B 169*	A. B 204	K. NY**
K. NY (type B)	—	+++	+++	+++
.....	A. B 169	—	—	+++
		A. MA 73	A. B 1076	K. F10
K. F10 (type C)	—	+++	+++	+++
.....	A. MA 73	—	—	—
.....	A. B 1076	+	—	++

*: A. stands for *Aerobacter*. **: K. stands for *Klebsiella pneumoniae*.

Table no. 3.
Representative results of complement fixation tests with mucoid organisms.

Antigen	Immune sera					
	Type A	Type B	Type C	Type D	MB 700 (<i>E. coli</i>)	MA 248 (<i>E. coli</i>)
Type A	192+	384+	96	0*	48+	384
Type B	192	384+	192—	0	48+	48+
Type C	192	384	96—	0	48+	192+
Type D	192	384	96—	192	96—	96+
Type AE	192	768—	96—	96	96+	384—
MB 700 (<i>E. coli</i>)	384+	768—	96+	48—	1536+	96+
MA 567 (<i>E. coli</i>)	192+	192+	96—	12+	384—	192—
MA 248 (<i>E. coli</i>)	192+	192+	48+	12+	384	1536
B 1076 (<i>Aerob.</i>)	96+	96+	48+	0	96+	96+
B 169 (<i>Aerob.</i>)	96—	384—	48+	48—	96—	96+
MA 73 (<i>Aerob.</i>)	96	192+	48	0	96—	48+
S 57-P (?)	192+	768—		0	48+	192—

*: 0 indicates negative reaction in highest concentration of serum (1:12).

A + following the titer indicates partial fixation (more than 50 % hemolysis) in the next higher dilution. A — indicates partial (less than 50 %) hemolysis at this serum dilution.

Only the results with one strain of each sero-type is given. Tests with other strains of the same types gave similar results.

Table no. 4.
Agglutination tests with autoclaved antigens.

Serum	Antigens											
	Type A		Type B		Type C		Type D		Type AE		S 57-P	
	I	II	I	II	I	II	I	II	I	II	I	II
Type A	2560	++	20		0		20		160		0	
Type B	160		2560	+++	40		0		40		0	
Type C	40		40		320	++	0		40		0	
Type D	0		0		0		80	+	0		0	
Type AE	40		0		0		1280		320	—	20	
MB 700 (E. coli)	0		0		0		0		40		0	1280
S 57-P	0		0		0		0		40		80	

Column I gives the agglutinin titers with the autoclaved antigens, column II results of precipitin tests of the supernates of the autoclaved antigen (free, serologically active capsular substance).

All strains produced acid from glucose, and all but one also from maltose and mannitol (the one exception seemed to be a similar organism as *Klebsiella paulum fermentans* of ten Have (1913)). Otherwise fermentation reactions were variable. Slow lactose fermenters and lactose-negative strains were found in all species. The behaviour of a number of strains, both of *E. coli* and *K. ozaenae*, on bromthymol blue lactose agar plates, indicated that these strains may often be mutabile types as several strains produced acid (yellow) spots in the alkaline (blue) growth, or yellow secondary colonies, which were sometimes raised above the level of the non-fermenting growth. Subculture of these secondary colonies always yielded rapid lactose fermenters.

Of 31 strains of *K. ozaenae* only 5 fermented lactose in 24 hours, the others required from 2 to 10 days or remained negative after 10 days (6 strains).

Out of 10 strains of *K. pneumoniae*, one type A strain was negative after 10 days, another positive after 2 days. The others fermented lactose within 24 hours.

Saccharose was fermented by 7 of 31 strains of *K. ozaenae* (usually delayed fermentation), and by all strains of *K. pneumoniae* within 24 hours.

Gas production in glucose was variable, absent in 19 out of 31 strains of *K. ozaenae* and in one strain of *K. pneumoniae* type A.

The methyl red reaction was positive in all strains of *K. ozaenae* and *K. pneumoniae* types A and C, negative in one of the type B strains.

The Voges Proskauer reaction was negative in *K. ozaenae* and *K. pneu-*

moniae type C, positive in *K. pneumoniae* types A and B (when the usual NaOH reagent was used, only some of the strains showed a slightly positive reaction but by use of α -naphthol the reactions became strongly positive).

Only a few of the ozaena strains produced H_2S in lead acetate agar.

In Koser's citrate medium only the strains of *K. pneumoniae* types A and B and some of the strains of *K. ozaenae* were able to grow.

Nitrates were reduced by all but 5 strains, gelatin was not liquefied and no *Klebsiella* strains produced indol or showed motility.

The mucoid strains of *E. coli* showed all the typical reactions of this species, including indol formation. Only 3 of the strains were motile.

Finally the 4 *Aerobacter* strains, which cross-reacted in type B or type C immune serum, gave a marked H_2S reaction in lead acetate agar, a positive Voges Proskauer reaction, two of them a positive, the others a negative methyl red reaction. They were able to utilize citrates and did not produce indol. They were non-motile.

Discussion.

Typing by slide agglutination is a simple, quick and apparently quite reliable methods and seems to give all the information that is required. Reactions are very strong and convincing. Only in certain instances of cross-reactivity may the results be misleading. In such cases cross-absorption tests may become necessary.

By using a very limited number of different immune sera it was possible to determine the sero-type of all the mucoid strains that were studied, with the exception of 3, which lost their capsules before the examination was complete. These strains appeared to be strains of *E. coli*.

The fact that the majority of the *Klebsiella* strains isolated in this laboratory were type D strains is in good agreement with the results of the Dutch workers at the Instituut voor Tropische Hygiene in Amsterdam. The results show that the majority of saprophytical — or slightly pathogenic — *Klebsiella* strains found in the respiratory tract belong to this type, not only ozaena strains, but strains from asthma, chronic bronchitis, chronic rhinitis and other diseases of the respiratory tract. It is very likely that these strains mostly live in the nose, and that they may often only be contaminants from the nose when found in sputum or other material. Only 4 strains, isolated in this laboratory, could be classified as *K. pneumoniae*, and they were all type A strains. It is significant that the only strain from a severe infection belonged to this type. It is quite clear, also, that this organism, although it is the most strongly pathogenic type may, not infrequently, be found as a saprophyte in the respiratory tract.

Types B and C were not represented in our material, but it is of some interest that the *Aerobacter* strains isolated from urinary infections gave marked cross-reactions with one or the other of these types. The differentiation between *K. pneumoniae* type B and *Aerobacter* strains of the same serological reactivity is very difficult, and

may be impossible. The main reason for the differentiation in this case was that differences could be found between the polysaccharides of our two strains and that of type B.

Of the remaining sero-types, only ten Have's type AE was represented in this material, but no strains belonged to the types E or F.

Finally one strain biochemically and culturally seemed to correspond to ten Have's *Klebsiella paulum fermentans*, the relationship of which to the other *Klebsiella* species is so far unknown. No type strains were available for comparison.

The results of the studies reported in this paper are in full agreement with the results of Goslings (1933), Goslings and Snijders (1935), Wielenga (1937) and ten Have (1943), at least as far as capsular types and cultural and biochemical properties are concerned. It seems that there is every reason to support the opinion of these workers that *Klebsiella* can be divided into 3 distinct species, *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis*. It usually appears to be easy to distinguish between these species and between them and other related organisms, with the exception of the differentiation between *K. pneumoniae* type B and *Aerobacter*.

The results reported here seem to justify the statement that *K. ozaenae* type D, the most common *Klebsiella* type in the respiratory tract, has a very low pathogenicity, if any. Thus only one of our strains of *K. ozaenae* was isolated under circumstances which suggested its pathogenic importance, namely from the inside of a maxillary sinus. This strain, however, proved to be the only representative of sero-type AE, and the sinus at the same time contained a pneumococcus and the strain thought to be *K. paulum fermentans*, so even the pathogenic role of this strain is questionable. All the others were isolated from cases where the pathogenic role of the organism seemed very questionable. This leads to the conclusion that the isolation of this, most common type of *Klebsiella* from pathological material usually has very slight significance as far as the causation of infections is concerned. The possibility remains, however, that these organisms may do some harm e. g. by causing hypersensitivity in the patient. Such cases have been reported (e. g. Henriksen 1937).

There seems to be little reason to discuss the properties of the *Klebsiella* species and types in further detail in this paper, as such discussions have been published already by Goslings (1933), Wielenga (1937) and ten Have (1943). All these authors studied much larger collections of strains than our's. As the conclusions of these Dutch workers do not seem to have received official recognition (vide Bergey's Manual 1948), these questions will be dealt with in more detail in a subsequent paper.

It was something of a surprise that all the strongly mucoid urinary strains of *E. coli*, that could be typed at all, showed strong reactions in the same immune serum. The results obtained by various authors,

quoted in the introduction, suggests a great heterogeneity of the coli group with respect to capsular antigens. It is possible that this very strongly mucoid type of colony may be characteristic of some special K-type (our strains have not been tested in any of the known K type-sera). It is also possible that this mucoid form may not be entirely analogous with the so-called capsular form, first described by Kauffmann, and later studied extensively by Vahlne (1945) and others. It should be mentioned that Kauffmann (1943) states that all mucoid strains were excluded from his material. It has not been shown that all our mucoid strains of *E. coli* had an identical capsular polysaccharide. It is possible that it may have been a question of cross-reactions only, but at least they have some common antigenic factor. Further studies of these strains are in progress. So far it has been difficult to produce good immune sera against these capsular antigens, and only one of a number of rabbits produced a really satisfactory serum. Tests with some other, very weak sera seemed to give similar, although not equally convincing results. It may be mentioned here that studies of O-antigens of these strains has shown that they belong to different O-groups (only two strain isolated from different patients had the same O-antigen). Thus there is no question of repeated isolation to the same, possibly particularly pathogenic, organism from all these patients.

Summary.

By slide agglutination 48 out of 51 strains of encapsulated gram-negative rods from pathological material could be referred to one of 7 sero-types. The slide agglutination seemed to give entirely type-specific reactions. 36 strains, all but one (a case of liver abscess) isolated from the respiratory tract, were classified as *Klebsiella*. 30 strains were *K. ozaenae* type D, one type AE and 4 *K. pneumoniae* type A. One strain might possibly be *K. paulum fermentans* (ten Have).

K. ozaenae, in particular type D, seems to have a very slight pathogenic significance.

Of 12 strains from other sources (mostly urinary infections). 8 strongly mucoid strains of *E. coli* reacted strongly in the same immune serum, and seemed to have related or identical capsular substances. Of 4 strains of *Aerobacter* 2 gave very strong cross-reactions with *K. pneumoniae* type B and 2 with type C.

Separation of the different species or types by complement fixation appeared to be impossible. Sera against *K. ozaenae* type D seemed to give species-specific reactions with *K. ozaenae*, sera against *K. pneumoniae* gave unspecific group-reactions with *Klebsiella*, *Aerobacter* and *Escherichia*, whereas *E. coli* immune sera gave strong reactions

with strains of the same O-group, and weaker group-reactions with other organisms.

Autoclaved *Klebsiella* antigens remain type-specific, but become more easily agglutinable.

Studies of cultural and biochemical properties of the *Klebsiella* strains gave results which agree completely with those published from the Instituut voor Tropische Hygiene in Amsterdam.

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TAXONOMY AND CLASSIFICATION OF THE GENUS KLEBSIELLA

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The taxonomy and classification of encapsulated gram-negative rods belonging to genus *Klebsiella* seem to remain confusing in spite of numerous careful and extensive studies. Some authors have voiced the opinion that these organisms are nothing more than mutants of other members of the tribe *Eschericheae*. According to this trend of thought a separate *Klebsiella* genus would hardly be justified. (Parr 1939).

Others claim that it is practicable and useful to distinguish between *Klebsiella* and other genera, and even to distinguish between different species of *Klebsiella*. The majority of those who have worked with these organisms within the last 20 years support the latter opinion.

The treatment of the genus *Klebsiella* in the two latest editions of Bergey's Manual of Determinative Bacteriology (1939, 1948) reflects the uncertain status of the genus. Whereas the 5th edition lists 6 different species, the 6th edition radically cuts down the number to a single species and reduces the rest to the rank of foot notes.

It is true that some of the species listed in the 5th edition (*K. capsulata*, *K. paralytica*, *K. granulomatis*) were of very questionable validity, and possibly only represented one of the other species under different names. At least no reliable criteria for the identification of these species were available apart from biochemical reactions of very limited value. Thus a certain degree of simplification was clearly justified. With respect to the three other species, however, a very considerable knowledge has been accumulated through a series of studies, and one may question the justification of such a radical change as in the 6th edition of the manual. The reduction of the genus to a single species seems to have had unfortunate consequences for the identification key, which, in its present form, seems to be of limited usefulness in practical diagnostic work.

Any one who attempts to use this key in the identification of a strain of encapsulated gram-negative rods will find that it gives no clue as to how to distinguish between strains of *Klebsiella* and mucoid strains of *Escherichia* or *Aerobacter*. As a matter of fact the list of characteristics of *K. pneumoniae* is a practically complete list of the properties of the two other genera of the tribe. A few quotations are given to prove this statement:

»Litmus milk variable. Indole variable, usually not formed. Fermentation of carbohydrates highly variable. Acetylmethylcarbinol production variable. Methyl red test variable. Utilization of citrate as a sole source of carbon variable. Habitat: associated with infections of the respiratory, intestinal and genito-urinary tracts of man. Encountered in infections of animals and may be isolated from a wide variety of sources.«

Finally, the fundamental works of Julianelle are only mentioned in a foot note, and no mention at all is found of the many very careful studies which have originated in the Institut voor Tropische Hygiene in Amsterdam. If the quotations given above represented the sum of our knowledge of these organisms, it is somewhat difficult to see how this genus could be valid at all.

A survey of the literature of the last 20 years (and some personal experience with these organisms) have convinced the author that it is possible to arrive at a better system of classifying this genus and its species. The present key places the main emphasis on cultural and biochemical properties, which are of secondary importance in classification, and it pays only slight attention to characteristics which, in the opinion of experienced workers, are of essential value, viz. habitat, pathogenic properties and antigenic structure.

The earlier works on these organisms, although containing valuable information, are of limited value to this discussion, partly because many early workers only studied a very limited number of strains, partly because they had no reliable means of classification. A perusal of some of these early works makes it obvious that the identity of many strains may have been questionable, and that mucoid strains of *Escherichia* or *Aerobacter* may have been included in some collections. It was not realized fully in the early days that many gram-negative rods may appear in the mucoid phase, nor that mucoid strains of e. g. *E. coli* are quite common.

I. *Antigenic structure.*

A. *Capsular antigens.* The demonstration by Julianelle (1926, 1930) of specific sero-types within a collection of strains mainly isolated from pneumonia and other severe infections, established the fact that these organisms could be divided into the types A, B and C and a heterogeneous group X. Such convincing evidence in support of this was brought forward by Julianelle himself and by Heidelberger, Goebel

and Avery (1925) and Goebel and Avery (1927), that there could be little doubt about the validity of the results. The results have, furthermore, been confirmed by Goslings (1933), Goslings and Snijders (1936), Prasek and Prica (1933), Wielenga (1937), Kurylowicz (1938), ten Have (1943) and the author (1949). A few workers such as Elherl and Gerkess (1929) and Prica (1930) expressed differences of opinion with Julianelle, but on insufficient grounds as they had not then employed Julianelle's methods. Prica later changed his opinion.

Goslings (1933) and Goslings and Snijders (1936) showed that a large number of rhinoscleroma strains all belonged to a single serotype, the capsular polysaccharide of which was serologically indistinguishable from that of Julianelle's type C, but might possibly be distinguished from it by phage typing. Similar results were obtained by Prasek and Prica (1933), Morris and Julianelle (1934), Wielenga (1937), Kurylowicz (1938) and ten Have (1943). Kurylowicz confirmed the observation that certain phage strains had a different action on *K. pneumoniae* type C and *K. rhinoscleromatis*. On the other hand M. L. Rakieten, Eggerth and T. L. Rakieten (1940) isolated phage strains which were equally active against both organisms. According to ten Have 128 strains of *K. rhinoscleromatis* were all serologically identical.

Typing of ozaena strains was carried out successfully by Goslings (1933) and Goslings and Snijders (1936). The results were confirmed by Julianelle (1935), and by Wielenga (1937) and ten Have (1943), who found new types that were not represented in Goslings' material. So far the types D, E, F, AE and C have been established among ozaena strains. The type AE had partial antigens in common with both types A and E, and type C has, so far, only been found among strains from the East Indies. Only occasional strains could not be typed (two out of 330 strains). Type D was by far the most common. The latter result was confirmed by the author in a collection of strains isolated in Norway (1949). The author has found no single paper that throws any doubt on these results.

Consequently it may be safe to consider it as a fact that the following sero-types exist among *Klebsiella* strain:

In *K. pneumoniae* A, B and C, in *K. rhinoscleromatis* C' and in *K. ozaenae* D, E, F, AE and C. The actual nature of the X strains of Julianelle is partly unknown. Some were found to belong to *K. pneumoniae* by study of the somatic antigens. Others may possibly have been strains of *K. ozaenae*. Ten Have (1943) was able to type all the 37 strains of *K. pneumoniae* at his disposal, and found no X strains.

B. *Somatic antigens.* Julianelle (1926 b) showed that capsule-free strains of *K. pneumoniae* had a common somatic antigen, regardless of original type. Goslings (1933) and Goslings and Snijders (1936) showed that each of the species *K. pneumoniae*, *ozaenae* and *rhinoscleromatis* had a separate species-specific antigen. These antigens showed

marked cross-reactions with those of the other species and of *Aerobacter*, but could be shown to have species-specific components by the agglutinin titers and by absorption. Similar results were obtained by Elbert and Gerkess (1929), Kurylowicz (1938), Price (1930). Ten Have (1943) finally presented results of examination of a large number of strains, and confirmed these results. In *K. ozaenae* he found that occasional capsule-free strains were less specific than the majority, and gave very strong cross-reactions with *K. rhinoscleromatis*, but he had no difficulties in the identification of such strains.

Although the results of studies of somatic antigens are less clear-cut than of capsular antigens, they appear to the author to be convincing — at least as convincing as much other evidence which is used in the classification of bacteria. Although it may not be absolutely necessary to utilize these results in the classification, they are unquestionably of great importance in a few rare cases.

II. *Habitat.*

It is evident that all *Klebsiella* types, which have been properly studied and identified, with the exception of *K. pneumoniae* type B, practically exclusively occur in the respiratory tract. Exceptions to this rule have only been reported occasionally.

K. pneumoniae is first and foremost a specific cause of pneumonia in man, but there are certain differences between the three types.

Type A. Of 42 strains studied by Julianelle (1930) 40 were isolated from man, 33 from cases of pneumonia, 1 from a liver abscess and 5 were saprophytes in the respiratory tract. Only 1 strain was isolated from urine, and two in abscesses in guinea pigs. Of ten Have's (1943) 11 strains 5 were isolated from cases of pneumonia, 4 from the nose and 1 from peritonitis. The author (1949) isolated 1 strain from a case of liver abscess and 3 from nose or sputum in chronic diseases. The author has been unable to find a report of the isolation of this organism from any other source than man or animals.

Type B. 7 of Julianelle's 12 strains were isolated from infections in animals. The rest were found in human pneumonia (2), throat, liver abscess and feces. Edwards (1928) also found this organism in infections in animals. Finally ten Have isolated his 11 strains from pulmonary disease (2), pneumonia in a guinea pig, throat (2), pleura, osteomyelitis, peritonitis, urine and feces.

Type C is less common. Julianelle found 3 strains in human pneumonia, and one each in a sinus and a nose. Ten Have found 2 strains in pneumonia and 9 in the nose in ozaena. Thus this organism has not, apparently, been found outside the respiratory tract of man.

K. ozaena. The different types are found in the same places. The overwhelming majority of strains are found in ozaena. Other strains have been found in the sputum in chronic bronchial diseases, in throat, eyes, and occasionally in sinusitis. Only very few strains have been

found outside the respiratory tract. The majority of strains were found in cases of chronic diseases, and very few, if any, have been shown to be the cause of severe acute infection.

This has been shown very clearly by ten Have. The author (1949) found that a number of strains behaved as stated by ten Have.

K. rhinoscleromatis. There is no report, as far as the author knows, of the isolation of this organism in any other cases than rhinoscleroma patients or contacts. In rhinoscleroma it is found practically constantly.

By comparing the serological properties and habitat of these microbes, there can be little doubt that they behave as biological entities. This is further strengthened by the pathogenic properties.

III. *Pathogenicity.*

K. pneumoniae is a fairly important and dangerous pathogen. Types A and C are particularly pathogenic to man, especially type A, which is the most common cause of Friedländer pneumonia. Type B is, perhaps, more frequently pathogenic to animals, but it also causes diseases — not quite strictly limited to the respiratory tract — in man. Types A and B are highly virulent to mice, type C non-pathogenic. *K. ozaenae* seems to be a comparatively harmless saprophyte (unless it actually causes ozaena) and is practically never found as the cause of serious disease in man or animals. Its virulence for mice is low or it is non-pathogenic (Julianelle 1935).

K. rhinoscleromatis probably causes rhinoscleroma, and no other disease. It is non-pathogenic to mice. (Morris and Julianelle 1934).

These properties, then, form the base of the classification of these organisms. Also cultural and biochemical characteristics may be of value, and may be utilized as means of identification, but in some cases the results are inconclusive. Still a list of the characteristics of each separate species clearly brings out the differences between them.

IV. *Morphological and cultural characteristics.*

There is little to add to the classical descriptions. Only one point should be mentioned, viz. the appearance of the colonies.

To the experienced worker a difference between the colonies of the different species usually is of some value, as pointed out by Goslings (1935) and ten Have (1943). The author has had the same experience (1949). *K. ozaenae* types D, and F and *K. rhinoscleromatis* grow on agar plates with flat, watery, confluent colonies of a thin syrupy, more or less ropy consistency, and are fairly translucent, slightly greyish. *K. pneumoniae* and *K. ozaenae* types E and AE grow in domed, comparatively firm usually very viscous colonies. (In the experience of the author the strains of types B and C were more opaque, dirty white and of a soft butyrous consistency, whereas type A was more trans-

parent and more viscons, but as only two strains each of types B and C were studied, this point needs confirmation). All authors are unanimous that genuine *Klebsiella* strains are non-motile and encapsulated.

V. Biochemical reactions.

A. Properties common to all *Klebsiellae*.

Gelatin never liquefied. Coagulated serum never liquefied. Indol reaction always negative (it is doubted that any authentic strain has been described which formed indol). Nitrates are reduced to nitrites by most strains (Goslings (1935) found a few exceptions in all groups).

B. Properties of differential value.

K. pneumoniae. Most strains ferment glucose, maltose, mannitol, lactose and sucrose with production of gas. Some strains are late lactose-fermenters or do not ferment it. Some strains fail to produce gas. (Julianelle 1930, Goslings 1935, ten Have 1943, Henriksen 1949). Amygdalin is fermented. Milk usually coagulated (sometimes only after boiling (ten Have)). Litmus is reduced in 1 % glucose $\frac{1}{2}$ % agar medium. H_2S is not produced in cystine broth (Goslings 1935). Bile does not inhibit growth.

Type A. Appears to give a positive Voges-Proskauer reaction if a sufficiently sensitive method is used (ten Have 1943). Methyl red reaction variable (ten Have 1943, Henriksen 1949). Dulcitol is not fermented. Growth in Koser's citrate medium is weak or fails. Litmus is reduced in 1 % glucose $\frac{1}{2}$ % agar medium (ten Have 1943).

Type B. Gives positive Voges-Proskauer reaction if sensitive method is used. Methyl red reaction variable. Dulcitol is not fermented. Good growth in Koser's citrate medium. Litmus is reduced.

Type C. Voges-Proskauer reaction negative. Methyl red test positive. Dulcitol is fermented. Good growth in Koser's citrate medium. Litmus reduced by some strains.

K. ozaenae. All strains ferment glucose, maltose and mannitol. Most strains are late lactose-fermenters, and some do not ferment lactose. Saccharose is fermented by some strains, not by others, and in many cases only after 2 to 14 days. Many strains produce only traces of gas or none at all.

Amygdalin is fermented by some strains. Milk coagulated by some strains. Litmus is not reduced in 1 % glucose $\frac{1}{2}$ % agar medium. H_2S is produced in cystine broth by some of the strains. Bile inhibits the growth of some of the strains. Some strains are able to utilize citrate as sole source of carbon. Voges-Proskauer reaction always negative, methyl red test always positive (sometimes weak). Dulcitol not fermented.

K. rhinoscleromatis. This species has much more uniform biochemical reactions than either of the other species. (Goslings 1933, Prica 1930, Elhert 1929 b, Morris and Julianelle 1935, Wielenga 1937,

ten Have 1943). Glucose, mannitol and maltose fermented by all strains. Saccharose always fermented, but often only after 3 to 7 days. Lactose is never fermented within 4 days. After 7 to 14 days most strains produce acid (Goslings 1935). Gas is never produced, amygdalin not fermented, milk not coagulated, litmus not reduced and H_2S not produced in cystine broth. Bile inhibits growth. Acetylmethylcarbinol is not produced. The methyl red reaction is positive. Dulcitol is not fermented. No strain is able to utilize citrates as sole source of carbon.

It may seem as if these biochemical reactions are too variable to be used in the identification, but actually it is possible to arrive at a diagnosis, on this basis only, in the majority of cases.

K. pneumoniae is distinguished from *K. ozaenae* and *K. rhinoscleromatis* by the positive Voges-Proskauer reaction (types A and B), or the fermentation of dulcitol (type C). *K. ozaenae* can practically always be distinguished from *K. rhinoscleromatis* by one or more of the following reactions: fermentation of lactose within 4 days, fermentation of saccharose, gas production, ability to grow in bile, ability to utilize citrate as sole source of carbon. Ten Have (1943) found that only two strains of *K. ozaenae* out of 330 agreed with *K. rhinoscleromatis* on all these points, and consequently had to be identified by serological means. Even the 3 types of *K. pneumoniae* can usually be distinguished from each other comparatively easily by the following tests: Growth in citrate medium (type A negative or slightly positive, types B and C positive), fermentation of dulcitol (A and B negative, C positive) and the Voges-Proskauer reaction (A and B positive, C negative).

The greatest problem in identification apparently is to distinguish between *K. pneumoniae* type B and *Aerobacter aerogenes*, as these species appear to be closely related.

Other species of Klebsiella.

A number of other »species« of *Klebsiella* have been described, mostly in older literature. There is good reason to believe that most of these actually are the same as one or the other of the 3 species that have been described above. Thus ten Have (1943) identified a strain of *B. mucosus* Fasching as *K. pneumoniae* type B, and a strain of *B. mucosum* Howard as *Aerobacter aerogenes*.

With respect to some such species, there is a possibility that they might be separate species (*K. granulomatis*, *K. paralytica*) but the available evidence is insufficient, and it is consequently not justified to create separate species for these organisms. Finally it should be mentioned that ten Have (1943) described a new species, *K. paulum* fermentans, mostly characterized by lack of biochemical activity. Some strains fermented glucose with acid production, whereas others fermented no carbohydrate. The strains could be divided into two

serotypes, I and II. There was no correlation between sero-type and ability to ferment glucose. The relationship of these strains to the other *Klebsiella* species is not yet clear, as no capsule-free mutants could be isolated. The author has only seen one strain that might fit the description, and therefore does not want to express an opinion as to whether this should be recognized as a new species.

Suggestions for a new key to the genus Klebsiella.

It is believed that Habitat, pathogenic properties and antigenic structure should form the basis of classification.

A revised description of the genus, and key to the identification of the species is suggested below:

Genus III Klebsiella Trevisan.

Short, often plump rods with rounded ends, occurring singly or in pairs. Encapsulated. Non-motile. Do not liquefy gelatin or coagulated serum. Usually reduce nitrates to nitrites. Do not form indol. Aerobic and facultative anaerobic, growing well on ordinary culture media. Encountered principally in the respiratory tract of man.

Key to the species of genus Klebsiella.

I. Associated with pneumonia and other acute inflammations of the respiratory tract or with acute infections in animals. Capsule-free strains possess species-specific antigen.

1. *Klebsiella pneumoniae*:

- a. Type-specific polysaccharide A.
1 a. *Klebsiella pneumoniae* type A.
- b. Type specific polysaccharide B.
1 b. *Klebsiella pneumoniae* type B.
- e. Type-specific polysaccharide C.
1 e. *Klebsiella pneumoniae* type C.

II. Found in ozaena and in other chronic diseases of the respiratory tract, probably mostly saprophytical. Capsule-free strains possess species-specific antigen.

2. *Klebsiella ozaenae*.

- d. Type-specific polysaccharide D.
2 a. *Klebsiella ozaenae* type D.
- e. Type-specific polysaccharide E.
2 b. *Klebsiella ozaenae* type E.
- f. Type-specific polysaccharide F.
2 c. *Klebsiella ozaenae* type F.
- g. Type-specific polysaccharide AE.
2 d. *Klebsiella ozaenae* type AE.
- h. Type-specific polysaccharide C.
2 e. *Klebsiella ozaenae* type C.

III. Associated with rhinoscleroma. Exclusively found in patients

suffering from this disease or their contacts. Capsule-free forms possess species-specific antigen. All encapsulated strains have the same type-specific polysaccharide (C').

3. *Klebsiella rhinoscleromatis*.

This key would then be followed by the description of the species, and of the types in cases where the types have different properties (*K. pneumoniae*). There is no need to repeat here the characteristics which have already been summarized. Other reactions may, if desirable be found in the literature, particularly in the works by Goslings (1933), Wielenga (1937) and ten Have (1943).

Finally, species of uncertain validity might, as usual, be mentioned in foot notes, viz. *K. paulum fermentans*, *K. granulomatis* and *K. paralytica*. It would do no harm, probably, to leave out all mention of all the other specific names from the old literature, which have a historical interest only.

Summary.

On the basis of the literature of the last 20 years and on personal experience, the author finds the treatment of genus *Klebsiella* in the latest edition of Bergeys Manual unsatisfactory. It is suggested that more attention should be paid to the contributions to our knowledge about this genus, which have been presented from the Instituut voor Tropische Hygiene in Amsterdam. A new description of the genus and a key to its species is suggested. The species *K. pneumoniae* (with the types A, B and C), *K. ozaenae* (types D, E, F, AE and C) and *K. rhinoscleromatis* (type C') should be recognized. Habitat, pathogenic properties and antigenic structure are the most reliable and useful criteria in classification.

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THE EFFECT OF SOME SPLITPRODUCTS OF 2,3'-AZOTOLUENE ON THE URINARY BLADDER IN THE RAT AND THEIR EXCRETION ON VARIOUS DIETS*)

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Since *Otsuka* and *Nagao* in 1936 reported that they had induced tumours in the bladder of rats by per-oral administration of 2, 3' azotoluene (AT) relatively few investigators have employed this azocompound. Instead, a comprehensive literature has grown up dealing with the azo-compound group, which produces tumours in the liver such as o-aminoazotoluene and p-dimethylamino-azobenzene (butter yellow).

Law (1941) gave subcutaneous injections to mice of altogether 5 mg. AT during the first two months and later implanted a 5 mg. pellet, when the animals were four months old. »No tumours involving the urinary bladder were observed but fibrosarcomas were induced following injection of 10 mg. of the dye in 5 of 20 mice«. *Kirby* (1945) mentions that *Seligmann* and *Shear* gave AT subcutaneously to mice without obtaining changes but that the finding of *Otsuka* and *Nagao* has been confirmed by *Cook* (personal communication).

Strömbeck (22) 1943—44 obtained hyperkeratosis and papillomatosis in 7 of 20 rats on a diet of rice flour, containing 20—30 mg. AT in the quantity of food consumed daily (ca. 10 g.). Previous experiments 1941—42 had been negative: 37 rats of the same stock were fed on a satisfactory diet — Hammarsten's diet 211 (1937) — (casein, rice, flour, sugar, arachis oil, cod-liver oil, yeast, wheat sprouts and salt mixture), and received 20 mg. AT as a 20 % solution in almond oil pipetted well down in the pharynx.

Strömbeck (22) 1943—44 implanted a segment of the bladder into the liver and then administered the azotoluene. While tumours deve-

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veloped in the bladder stump, the transplant in the liver remained normal, indicating thereby that the carcinogenic agent is contained in the urine. Hueper 1947 states that these experiments on rats seem to have settled the long controversy, whether the carcinogenic substances elicit the neoplastic responses by direct contact with the mucosa as urinary constituent or by the hematogenous way.

It thus seems natural to suppose that azotoluene or some of its metabolic products are to be found in the urine and from there act directly on the vesical epithelium. Preliminary experiments by Ekman and Strömbeck (see Strömbeck 21, 22) demonstrated that epithelial changes could be obtained by feeding rats on o- or m-toluidine in rice-diet. Analyses showed that azotoluene only occurs in small amounts in the urine.

It has been demonstrated that several azo-compounds are split in the azolink by the influence of yeast in vitro, (19) and that a corresponding splitting occurs in rats, when fed butter yellow Stevenson *et al.* (1942) or azobenzene Elson and Warren (1944). They could all undergo a benzidine type of rearrangement as assumed by Elson and Warren for azobenzene, the simplest member of the azo-group.

Thus it may be assumed from the theoretical viewpoint that AT is metabolized in the rat's organism to one or some of the following substances (Fig. 1).

o- or m-toluidine

2,3-methyl-benzidine

Demethylation of these can possibly give rise to aniline

benzidine

Oxidation processes among others may give

o- or m-aminobenzoic acid

4-amino-o-cresol or 4-amino-m-cresol

Acetylation and coupling reactions seem widely to occur in the aminogroup.

Ekman and Strömbeck (3) were able to show from these compounds in the urine of rats fed on azotoluene both aminocresol and m-amino-benzoic acid. It must, therefore, be supposed that toluidines also are formed in the organism.

I. Azotoluene and metatoluidine metabolism with varying diet.

In previous investigations (Ekman-Strömbeck 4) it could be demonstrated that the transition from the tumour-protective 211-diet according to Hammarsten (1937) to the tumorigenic rice diet resulted in changes in the metabolism of azotoluene. Thus rats fed on the 211-diet excreted greater quantities of oxidized decomposition products of azotoluene (aminobenzoic acid and aminocresol) than those fed on the tumorigenic diet, where increased amounts of toluidine were ex-

creted. As far as we are aware this is the first time that it has been demonstrated, that the excretion in the urine of the split products of a carcinogenic compound is influenced by dietary changes, which at the same time are of importance for the tumour frequency. Preliminary experiments pointed to riboflavine and casein being of importance. The results were connected with the simultaneous appearance of variations in the ascorbic acid synthesis, and in a later paper (*Ekman-Strömbeck* 5) it could be clearly shown that administration of both riboflavine and casein were required for a normal ascorbic acid synthesis in rats. Administration of casein alone did not change the ascorbic acid synthesis, but on the other hand the effect of riboflavine could not be produced without a simultaneous administration of casein. On ascorbic acid being given the excretion of m-aminobenzoic acid was increased in the same manner as on the administration of riboflavine (and casein). The experiments now put forward are intended to provide a continued study of the azotoluene metabolism against the background of the investigations previously referred to.

At first an investigation was made of the urinary output resulting from the administration of azotoluene in the doses which have given tumours of the bladder (30 mg. daily in oil solution by mouth).

Two groups of rats, I and II, each one comprising six animals, were placed on a basic diet consisting of 88 % vitamin-free wheat starch, 6 % peanut oil and 6 % salt mixture, to which an addition was made of 3 mg. aneurin and 200 mg. niacin per kg. of diet together with ca. 1 g. yeast per day. Thus the diet did not contain casein. After a few days the yeast was withdrawn from the diet in Group I. *Figures 2—4* show the excretion of ascorbic acid, m-aminobenzoic acid and aminocresol in both groups. Concerning determination of ascorbic acid, see *Ekman* (1). m-aminobenzoic acid and aminocresol were determined according to *Ekman* (2). As appears in figure 2 the excretion of ascorbic acid in Group II rises to 2—4 mg. per day. After 66 days this group also receives up to 22 % casein, which, however, does not result in any further increase of ascorbic acid excretion. Aminocresol and m-aminobenzoic acid likewise show (figure 3 and 4) an increased excretion in group II. For both these substances, however, the administration of casein results in an increase of the excretion, which is very pronounced in the case of aminocresol, but less so in the case of m-aminobenzoic acid.

The excretion of ascorbic acid in group I decreases after the yeast has been excluded from the diet, falling from the initial value of 2 mg., at the moment when the yeast is administered, to a value of ca. 0.25 mg. Thus the animals now only obtain niacin and aneurin with the basic diet. The excretion remains at this level even when the niacin is first withdrawn from the diet and later added again. A rise in the excretion of ascorbic acid to 0.5—0.6 mg., thus roughly the double, is

obtained when ca. 100 gamma riboflavine is given per day. If casein be also added, the values rise to 1—1½ mg., and after the administration of yeast the values will rise to 2—3 mg.

The excretion curves for aminobenzoic acid and aminocresol are considerably more uneven, but on the whole show a good parallelism with the ascorbic acid curve. As regards both these substances a decrease is also obtained, when the yeast is excluded from the diet and a low excretion on only aneurin and niacin being administered: ca. 1 mg. aminocresol and 0.75 mg. m-aminobenzoic acid every day. Administration of riboflavine gives no effect, or an uncertain one, whereas casein + riboflavine increase the excretion of aminocresol to 2—5 mg. and m-aminobenzoic acid to ca. 1½ mg. daily.

The addition of riboflavine is now withdrawn from group II, which thus receives aneurin, niacin and casein added to the basic diet. A sharp fall in the excretion of ascorbic acid and aminocresol is noticeable. The fall in m-aminobenzoic acid is also perfectly clear but not as fully pronounced.

Yeast is given to group I, which accordingly receives aneurin, riboflavine, niacin, casein and yeast. This results in increased excretion of ascorbic acid, but scarcely any further increase in aminocresol or m-aminobenzoic acid excretion.

A statistical control of the figures shows that for all substances and also for aminocresol, in which the daily values vary most from one day to another, there occurs a significant difference between the excretion values of the two groups both for the experimental period of the test days 9—65 as well as 66—100.

Figures 5 and 6 show a corresponding experiment, which was carried out with administration of m-toluidine instead of azotoluene. The setting up of this experiment is somewhat different but here also the object has been to demonstrate the effect partly of a simultaneous administration of riboflavine and casein, partly of either of these additions. The results in this experiment are the same as in the preceding one. If the test animals receive as addition to the basic diet only riboflavine a lesser increase is obtained in the excretion of all three substances examined, namely ascorbic acid and the oxidation products aminobenzoic acid and aminocresol. If the test animals, on the other hand, receive both riboflavine and casein, a more pronounced increase in the excretion is obtained.

Both at the administration of azotoluene as well as m-toluidine additions of adermin and pantothenic acid were also given. No effect on the excretion of the substances examined could, however, be noticed.

II. *The appearance of bladder tumours on feeding split products of azotoluene.*

As briefly reported (21, 22) o- and m-toluidine were each given in 1945 to 12 rats on rice diet. The dose initially consisted of ½ of the azotoluene dose (2 g. of a 7.5 % solution of toluidine in peanut oil

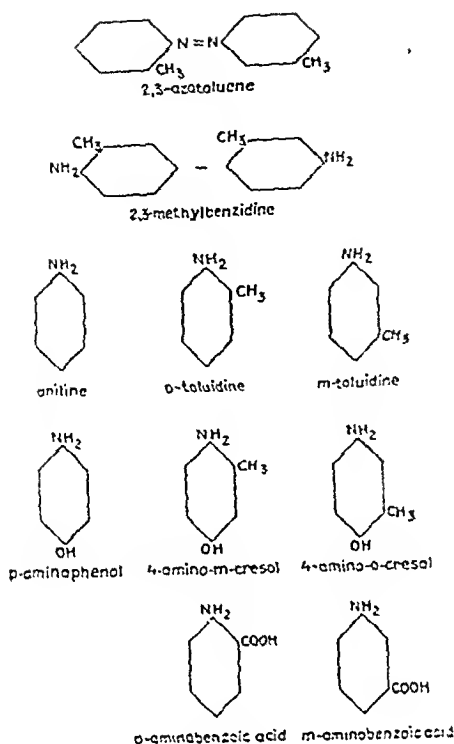


Fig. 1.
Azotoluene and some of its metabolites.

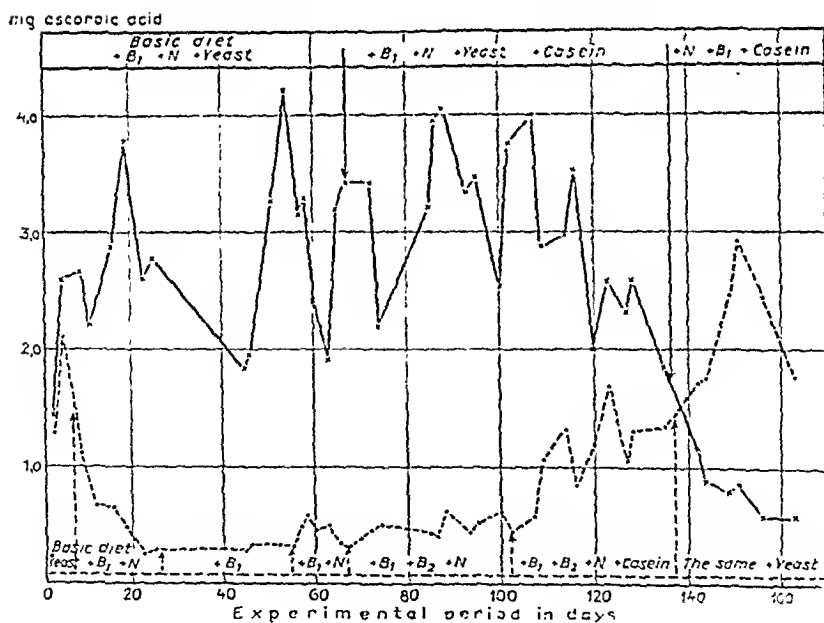


Fig. 2.

Output of ascorbic acid in the urine of rats which daily received 30 mg. 2,3-azotoluene by month. Each curve corresponds to the average values for 6 animals.

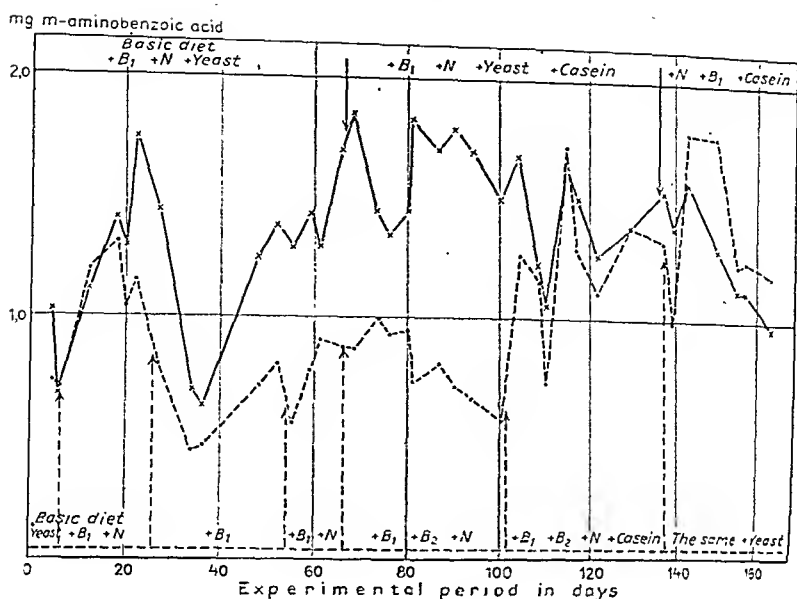


Fig. 3.

Output of m-aminobenzoic acid. Same experiment as in fig. 2.

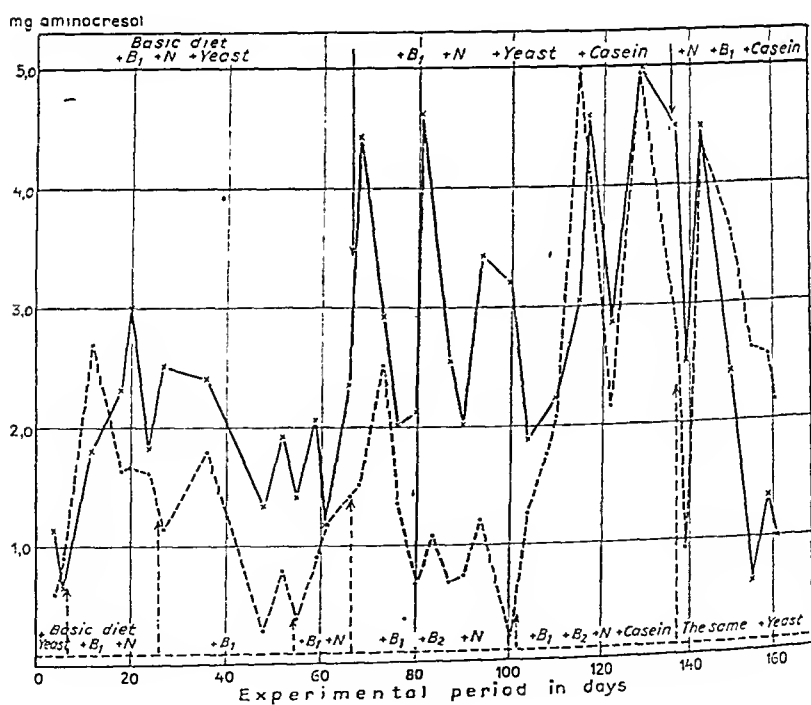


Fig. 4.

Output of aminocresol. Same experiment as in fig. 2.

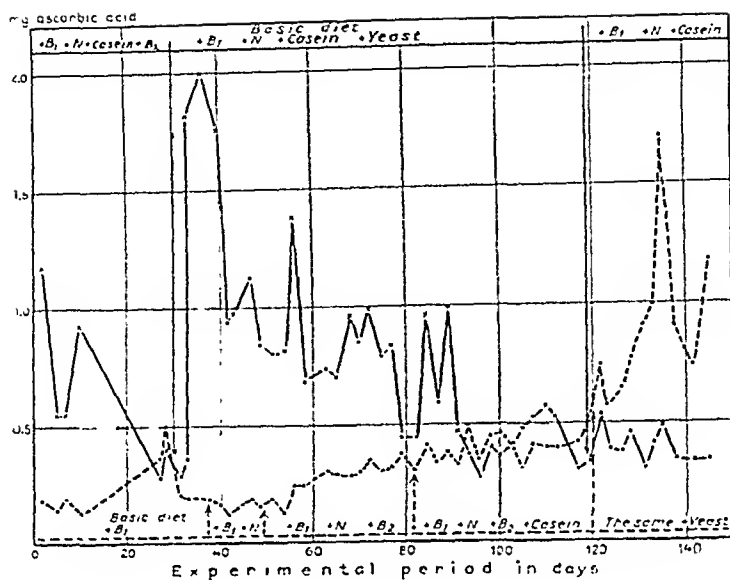


Fig. 5.

Output of ascorbic acid in the urine of rats which daily received 10 mg. m-toluidine by month. Each curve corresponds to the average values for 6 animals.

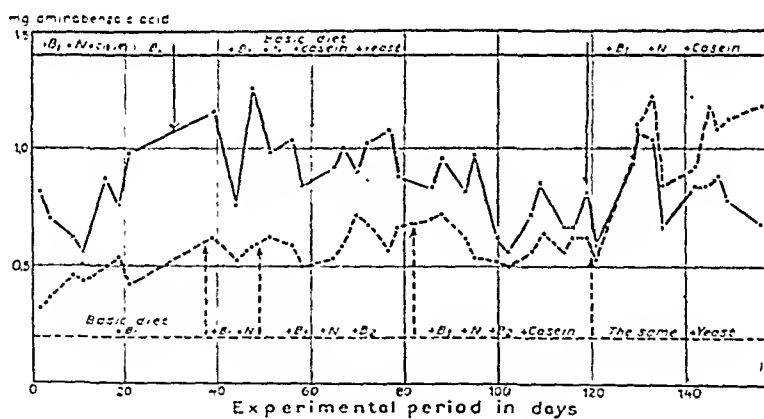


Fig. 6.

Output of m-aminobenzoic acid. Same experiment as in fig. 5.

were mixed with 98 g. rice flour), but had to be reduced after 64 days to half the size on account of weak general condition of the animals. All the time the animals received about 10 g. of carrot a week. The average weight in the *o*-toluidine series decreased from an initial 76 g. to 73 g., and in the *m*-toluidine series from 79 g. to 70 g. The survival period in the *o*-toluidine series was 91 days, in the *m*-toluidine series 95 days. Epithelial changes with keratosis, metaplasia and, in a number of cases, a tendency to incipient papillomatosis were found in 3 of the 10 bladders microscopically examined in the first series, and in 6 out of 9 in the latter series (see Fig. 4 in Strömbeck 21).

In a series of experiments, initiated in the summer of 1946, an investigation was made into the effect of *o*- and *m*-toluidine, *o*- and *m*-aminobenzoic acid together with *p*-aminophenol on the rat stock which had been used since 1941 for experiments on experimental bladder tumours. The results of this investigation are set out here. Experiments carried out simultaneously with aniline, which gave a positive result, are published in another paper.

When in 1946 *m*-toluidine was given in lower doses — 7.5–12 mg. daily — with the rice diet during 200–339 days to ten or so rats, the results could not be reproduced. The mucous membrane of the bladder was normal in all investigated cases. Nor were changes recorded, if the *m*-toluidine was added to the 211 diet. Possibly the dose employed here was too little. Variations may have occurred in the vitamin content of the rice flour, which was so difficult of access at that time.

In their investigations with *p*-dimethylaminoazobenzene the Madison group (8, 17) found that reproducible liver tumour frequency can be obtained on a diet, which contained the B-group's vitamins in quantities, lying at or somewhat under the minimum requirement and further containing corn oil (Table 1). On this diet the rat shows a

Table 1.
Basic diet 1.

Glycose (dextropur)	79 g
Casein (alc. extr.)	12 g
Salts	4 g
Corn oil	5 g
Riboflavin	0.2 mg
Thiamin-hydrochlor.	0.3 mg
Ca Pantothenate	0.7 mg
Pyridoxine hydrochlor.	0.2 mg
Choline chloride	3.0 mg
1 drop halibut liver oil per rat each month.	

<i>Saltmixture</i>	
Sodium chloride	52
Magnesium sulphate	116
Sodium phosphate, monobasic	104
Potassium phosphate, bibasic	286
Calcium phosphate, monobasic	162
Calcium lactate	390
Ferric citrate	35

certain growth and can be kept alive for at least a year. The importance of a diet not too deficient in vitamins has been emphasized by, among others, *Tannenbaum* (1947). His investigations showed that both transplanted tumours as well as tumours arising after a supply of different carcinogens, only attain full development when the animals receive diets allowing of a certain increase in weight and keeping them in good condition. This circumstance may have been the cause of the negative results with *m*-toluidine in 1945.

In a new series of investigations, initiated in June and September 1947, this synthetic diet (= diet 1) was therefore tested on 9 groups of animals, each group containing 12 rats of the same stock as had been previously used (as a rule 6 males and 6 females with an average weight of 167 g.). The substance to be tested was mixed directly into the diet. The toluidines were added to the diet in a quantity of 0.1 %, the oxidation products *o*- and *m*-aminobenzoic acid and *p*-aminophenol in a quantity of 0.2 %. Thus at an average consumption of 10 to 15 g. diet the daily supply becomes 10 to 15 mg. and 20 to 30 mg. respectively. Due to import difficulties corn oil could not be obtained in sufficient quantities and in the majority of groups had to be reduced to half the amount during the days 186 and 201, and excluded during the days 202—314. Only for the *o*-aminobenzoic acid group, which was started later, the corresponding periods were the days 91—106 and 107—219. No other oil was given as a substitute during these periods.

As already mentioned it has been shown (21, 22) that rats of the stock in use developed bladder tumours on administration of azotoluene in a rice diet, but not, on the other hand, in the complete 211-diet (9). The difference between these two types of diet consists among other things in that a sufficient content of vitamin B and protein are only found in the 211-diet. In later experiments (4) we found that the excretion of decomposition products of azotoluene could be affected by variations in the supply of riboflavin. Further experiments are set out in the first section of the present paper and seem to show that azotoluene metabolism is only influenced when riboflavin and casein are simultaneously administered. The riboflavin-casein effect can be assumed to depend on an indirect action on the ascorbic acid synthesis.

In order to demonstrate a possible connection between the effect of the diet constituents, partly on the metabolism of the carcinogenic compound, partly on the tumour frequency, we have, in the experiments with *o*- and *m*-toluidine, worked, moreover, with a series in which the animals instead of 0.2 mg. riboflavin per 100 g. diet, received 2 mg. (= diet 2), and with another series in which the animals daily received 20 mg. ascorbic acid by subcutaneous injection in addition to the basic diet (= diet 3). The basic diet contains 12 % casein and in these first experiments it was not considered suitable to change the addition of casein. On the whole the animals made an initial in-

crease in weight but then decreased somewhat as a rule. For reasons of space the animals were kept in boxes, 5—6 in number, and unfortunately a considerable amount of infection and cannibalism occurred on this relatively restricted diet.

Among the 9 groups bladder lesions with hyperkeratosis and epithelial proliferation of varying intensity developed in all groups except that of the p-aminophenol. (Some thickening of the epithelium was observed in one case of this group after 165 days. Stone of the bladder was present.) As control group 12 animals, simultaneously fed on basic diet without additions, have been jointly used for all series together with those of the following aniline paper.

Of the bladder changes four grades are schematically distinguished in the same manner as in our papers on aniline and acetaminofluorene.

Grade 1: Thickening of the epithelium and metaplasia to stratified squamous epithelium with cornification, yet without any tendency to proliferation to speak of. The changes in most cases strictly localized.

Grade 2: Extensive epithelial metaplasia with cornification and with proliferation, most often in the form of infolding and downgrowths of epithelial masses to the submucosa. Also smaller epithelial pearls with cornification may occur.

Grade 3: More pronounced epithelial proliferation with a tendency to formation of keratotic papillomas and often large cysts, containing keratotic masses.

Grade 4: More pronounced changes, possibly with atypical epithelium.

The results for the different groups are seen in the survey in Tables 2—10 and in Figures 7—12.

Epithelial changes of the renal pelvis have not been systematically searched for. In one rat, however, fed o-aminobenzoic acid (no. 7) cornification of the renal pelvis was found (grade 1, fig. 13). The renal epithelium was normal in 6 rats, exhibiting bladder changes of grade 2 to 4 after feeding of o-toluidine (2—4, 3—3), m-toluidine (1—4, 1—7, 3—11) and o-aminobenzoic acid (no. 12). Four rats fed m-toluidine (1—6, 2—2, 2—7, 3—8) showed normal epithelium as well of the bladder as of the renal pelvis.

In the control group (12 animals) epithelial changes occur in 2 out of 7 bladders investigated. Nevertheless, the changes have essentially less local extension than in the other groups and all belong to grade 1. They were entirely lacking in previous control material, in which 10 rats were fed on rice diet plus small quantities of carrot without addition of carcinogen.

About 50 % of the rats used in our investigations were infested with worms (*Trichodes crassicauda*). The fact that the parasites are found as frequently in all groups of rats even among the controls speaks against the suggestion that metaplasia and papillomatosis may be caused by this worm.

Table 2.

Bladder lesions in rats fed *o*-toluidine on diet 1.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1-01	M	276	160	220	+					—
1-02	M	36	150	165	+					—
1-03	M	172	165	210			+			—
1-04	M	175	150	140			+			+
1-05	M	11	205	—	+					—
1-06	M	178	180	155				+		—
1-07	F	276	165	170				+		—
1-08*)	F	213	180	165						—
1-09	F	276	165	160		+				—
1-10	F	276	160	190	+					—
1-11	F	276	175	190	+					—
1-12	F	276	170	180	+					—

*) not examined.

Table 3.

Bladder lesions in rats fed *o*-toluidine on diet 2.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
2-01*)	M	184	155	175						—
2-02	M	275	170	195				+		+
2-02	M	171	150	200				+		—
2-04	M	164	150	230					+	—
2-05*)	M	184	150	140						—
2-06*)	M	152	165	190						—
2-07	F	197	130	135	+					—
2-08*)	F	184	140	110						—
2-09*)	F	253	115	160						—
2-10	F	275	145	140	+					—
2-11	F	275	135	135		+				—
2-12*)	F	18	135	130						—

*) not examined because of cannibalism.

Table 4.

Bladder lesions in rats fed *o*-toluidine on diet 3.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
3-01*)	M	213	155	190						
3-02	M	172	180	240				+		—
3-03	M	265	185	200				+		—
3-04*)	M	175	165	190						
3-05	M	53	210	260	+					—
3-06	M	199	165	235	+					—
3-07	F	250	190	170			+			—
3-08	F	276	145	140	+					—
3-09	F	276	180	190	+					—
3-10	F	276	155	160	+					—
3-11	F	276	175	165	+					—
3-12*)	F	21	155	140						

*) no examination possible.

Table 5.

Bladder lesions in rats fed *m*-toluidine on diet 1.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1-01	M	255	200	175	+					—
1-02	M	258	205	200		+				—
1-03	M	251	190	170	+					—
1-04	F	341	150	130			+			—
1-05	F	259	160	160		+				+
1-06	F	341	145	170	+					—
1-07	F	257	160	165			+			—
1-08*)	F	107	155	195						
1-09*)	F	166	155	155						
1-10	F	148	150	125	+					+
1-11	F	140	155	155	+					—
1-12*)	F	319	145	200						

*) not examined.

Table 6.

Bladder lesions in rats fed *m*-toluidine on diet 2.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
2-01	M	252	195	210	+					—
2-02	M	279	160	200	+					—
2-03	M	241	215	205			+			+
2-04	F	256	185	180	+					—
2-05	F	83	140	120						
2-06	F	101	140	145						—
2-07	F	340	165	200	+					—
2-08	F	110	120	145						
2-09*)	F	368	105	165						
2-10	F	108	145	145	+					—
2-11	F	133	155	140		+				—
2-12**	F	330	155	170						

*) not examined.

Table 7.

Bladder lesions in rats fed *m*-toluidine on diet 3.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
3-01	M	244	200	195			+			+
3-02	M	132	185	205			+			+
3-03	M	135	220	180	+					+
3-04*)	F	134	140	140						
3-05*)	F	275	160	130						
3-06	F	147	140	125	+					—
3-07*)	F	269	140	135						
3-08	F	339	150	150	+					—
3-09	F	247	150	170	+					—
3-10*)	F	289	160	160						
3-11	F	265	135	170			+			—
3-12	F	257	190	145			+			—

*) not examined.

Table 8.

Bladder lesions in rats fed *p*-aminofenol on diet 1.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1	M	341	220	190	+					—
2	M	341	225	250	+					—
3	M	165	200	190	+					+
4	M	341	200	265	+					—
5*)	M	151	220	210						
6	M	193	185	220	+					—
7*)	F	341*)	195	210						
8	F	137	160	165	+					—
9	F	341	170	175	+					—
10	F	308	160	150	+					—
11	F	341	165	200	+					—
12	F	127	205	215	+					—

*) not examined.

Table 9.

Bladder lesions in rats fed *o*-aminobenzoic-acid on diet 1.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1	M	167	150	200		+				—
2*)	M	113	160	135						—
3	M	326	160	235	+					—
4	M	155	135	170	+					—
5	M	166	175	230	+					—
6	M	33	130	105	+					—
7	F	176	135	125				+		—
8	F	374	125	175				+		—
9	F	374	135	180	+					—
10	F	164	135	170			+			—
11	F	374	130	160	+					—
12	F	331	120	150				+		—

*) not examined.

Table 40.

Bladder lesions in rats fed *m*-aminobenzoic-acid on diet 1.

No.	Sex	Duration of feeding in days	Body weight (g)		Epithelial changes Grade					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1	M	137	230	215			+			+
2*)	M	131	240	215						
3	M	140	215	150			+			—
4*)	M	137	230	180						
5	M	130	205	230		+				+
6	M	140	175	175	+					—
7	F	262	150	135			+			—
8*)	F	180	160	195						
9	F	258	200	150			+			—
10*)	F	180	200	160						
11	F	84	160	115			+			+
12*)	F	222	160	135						

*) not examined.

It is known that deficiency of vitamin A in the diet produces hyperkeratosis in rats in the vagina, prostata, vesiculae seminales and also in the mucous membrane of the bladder (7, 10, 16). Hume, Burbank and Korenehevsky (1939) found that administration of estrogens to male rats gave severe changes of the bladder consisting of stratification, squamous metaplasia, keratinisation and papillomatosis, and that the most severe changes almost always occurred in those which were deprived of vitamin A and at the same time treated with estrogens. Their pictures show a striking similarity to ours and correspond to grade 2—3. Series of vaginal smears from our animals on diet 1 with addition of *m*-toluidine or *o*-aminobenzoic acid as well as, moreover, aniline and acetaminofluorene show to a great extent, however, a normal estrus cycle and thus do not point to any A-deficit occurring or suggest that the substances in question should, in this dose, have any estrogenic effect on the diet employed.

The administration of vitamin A has also, through the control analyses of the preparations given, been shown to attain at least 325 IU monthly. It can be stated with certainty that the administration of vitamin A, calculated per day, has attained at least 10 IU and most often more. This should satisfy the need more than enough.

On judging, however, the epithelial changes of the bladder in series 1—9, only grades 2—4, in which obvious epithelial proliferation occurs, should be reckoned as clearly pathological. Changes of this type (grade 2—4) occur thus:

Series 1		in 4 out of 11 bladders examined
2	o-toluidine	in 3 » » 6 » »
3		in 3 » » 9 » »
4		in 2 » » 9 » »
5	m-toluidine	in 1 » » 7 » »
6		in 4 » » 8 » »
7	o-aminobenzoic acid	in 3 » » 11 » »
8	m-aminobenzoic acid	in 5 » » 7 » »

These figures are too small for comparison of the frequency in the epithelial changes with feeding on different substances. Neither does the uncertainty in the division into grades make possible a safe comparison between the epithelial proliferation in the different series. If all the 6 toluidine series be combined, definite epithelial proliferation has been demonstrated in 17 out of 50 bladders investigated (34 %). In the two aminobenzoic acid groups epithelial proliferations have been found in 8 of the 18 investigated (44 %). In the former group grades 3—4 occurred in 7 out of 50 (14 %), in the latter in 3 out of 18 (17 %).

Ekman and Strömbeck (4) have put forward the assumption that the latent period of roughly 100 days needed for tumours to develop through azotoluene, can be shortened, if the test animals, before receiving AT, have received a diet devoid of protective factors. Part of the latent period may be necessary for the animals to attain the condition of deficiency which is obviously necessary for the development of pathological changes.

12 animals (8 males and 4 females) with initial weight of ca. 150 g. received Madison diet (= diet 1) without any addition of carcinogenic substance. After 139 days 0.3 % azotoluene was added to the same basic diet. The animals died 21—50 days after the beginning of the administration of AT. In 8 of them the bladder could be examined microscopically. In 7 of these 8 (AT during 41—50 days) were found epithelial changes of the mucous membrane. In at least 2 of these the changes already correspond to grade 2 (see *Fig. 14* and *15*). Yet another pair has almost the same development of the metaplastic keratotic processes with inflammation. In 3 it was a question of metaplasia without proliferation.

Thus it is striking that these changes developed after brief influence of AT. Moreover it is obvious that the substance showed a higher toxicity than otherwise.

Corresponding experiments have been carried out with m-toluidine. 12 animals with an average weight of 165 g. (8 males, 4 females) received diet 1 during 138 days, after which 0.1 % meta-toluidine was added to the same diet. Here the mortality was less, 4 died after 153 days of administration of m-toluidine. All had normal bladder epithelium. 2 died after 147 days and were not examined (cannibalism). Of

2, which died after 46 days, one had early epithelial changes (grade 1), one had normal bladder. The remaining 4 died after 21—50 days and could not be examined. These experiments are to be repeated.

The increased excretion of toluidines, which we observed on the absence of protective factors in the diet seems to indicate that it is the toluidines which should correspond to the real carcinogenic substance. Experiments to demonstrate a primary carcinogenic effect in toluidine have been made. To accomplish this, »pellets« were produced consisting of equal parts of paraffin or wax and toluidine (o- and m-) and with a toluidine quantity of 2.5—3 mg. in each pellet. The pellets have then been swept into the mucous membrane of the bladder, whereupon all of it has been implanted in the liver or m. pectoralis major in the same or in a related animal. Even after ca. 200 days no changes in the usually well preserved mucous membrane of the bladder could be observed.

Discussion.

The excretory experiments showed that in rats, which received azotoluene or m-toluidine, on a basic diet, completed with riboflavine-casein, the excretion of oxidation products, aminobenzoic acid and aminocresol, was increased. Any increased effect of the riboflavine-casein addition could not be obtained by adding yeast, adernin or pantothenic acid. Running parallel with this increase in the excretion appeared an increased excretion of ascorbic acid. In another paper (*Ekman and Strömbeck* (5)) it could be shown that after administration of m-toluidine the excretion of m-aminobenzoic acid, which in animals on a diet deficient in riboflavine-casein showed low values, could be increased by administration of ascorbic acid alone, and this would seem to indicate that the riboflavine-casein effect depends on an effect on the ascorbic acid synthesis.

The effect noticed here of riboflavine-casein on the metabolism of azotoluene and meta-toluidine can be compared with observations of *Kensler et al.* (1941), as well as of the Madison group, in experiments with p-dimethyl-amino-azobenzene according to which the frequency of liver tumours decreases on administration of riboflavine-casein. The metabolism of this carcinogen on different types of diet has not yet, so far as we know, been the subject of study. Riboflavine-casein as stated above, on the other hand, seems to act on the metabolism of the toluidines, which are carcinogenic for the bladder, in such a way that a lower frequency of bladder tumours should be the consequence, if the toluidines had a more powerful carcinogenic effect than their oxidation products. We have, however, not yet been able to give a direct proof that riboflavine-casein is the factor causing the lowering in the frequency of the bladder tumours observed in earlier experiments with azotoluene (21, 22).

No effect of the administration of riboflavine or ascorbic acid on

the tumour frequency could be observed in our experiments, when these vitamins were given as the only addition to the Madison basic diet. According to our own experience from excretion experiments a supply of both riboflavine and casein are required for an effect on the metabolism, and the same is true, as we find in other authors, for the effect on the development of liver tumours. It is possible that the content of casein in the Madison diet is not sufficient for the development of a riboflavine and ascorbic acid effect respectively. It has not yet been found possible to make this problem the subject of study in metabolical experiments.

In the experiments set out here the basic diet of the Madison group was necessary for the development of the epithelial changes. This diet seems to differ from other types of diet mainly by its content of corn oil. According to the Madison group the presence of corn oil in the diet promotes the development of liver tumours on administration of p-dimethylaminobenzene. Corn oil also seems, judging from our results, to promote the development of bladder tumours after administration of decomposition products of azotoluene. The same appears to hold good for acetaminofluorence.

As has already been mentioned the experiments showed that the excretion of the oxidation products aminobenzoic acid and aminocresol is increased after administration of riboflavine-casein, which can also be supposed to be a protective factor. It could be expected a priori that the oxidation products ought to have a lower carcinogenic effect than the toluidines. In the experiments set out here, however, the tumour frequency, on the administration of m- and o-aminobenzoic acid is not less than on the administration of the toluidines. Both the aminobenzoic acids were, however, administered in double doses compared with the toluidines and only new experiments with larger series and administration of both the types of substances in equimolar concentrations could decide the tumour frequency. The other oxidation product, which was found by us (4), namely aminocresol, has not yet been tested in morphological experiments.

Experiments have also been carried out with aniline and its oxidation product p-aminophenol. The result here was that aniline showed itself to have a cancerogenic effect, whereas this was lacking in p-aminophenol. Of the two oxidation products of azotoluene, aminobenzoic acid and aminocresol, it is, however, aminocresol above all which is comparable with p-aminophenol.

A very extensive work has been carried out to ascertain the mechanism of liver tumour development after administration of p-dimethylamino-azobenzene. A definite inhibition of tumour frequency by administration of riboflavine and casein has been found there. It would appear to be of essential importance that the same factors also affect the metabolism of azotoluene and its decomposition products. The tumorogenic effect in a series of decomposition products of p-

dimethylaminoazobenzene has been investigated, but all experiments have hitherto been negative. In our experiments, however, we have succeeded in obtaining carcinogenic effect from certain split products of azotoluene. Toluidines as well as aminobenzoic acids, namely, give tumours of the bladder during certain diet conditions.

Summary.

Some of azotoluene's theoretically imaginable split products (o- and m-toluidine, o- and m-aminobenzoic acid) were fed to albino rats of constant, but not systematically inbred laboratory stock as an addition to a synthetic basic diet with a low protein (casein) content, containing corn oil and of scanty vitamin content. All the substances tested here gave epithelial changes of the bladder with metaplasia, keratosis and papilloma entirely similar to those obtained on feeding with azotoluene the same stock of test animals. It should be noticed, however, that both aminobenzoic acids were administered in double doses compared to the toluidines. Concerning the latter no decrease in the frequency of the epithelial changes was obtained by the addition of further riboflavine or of ascorbic acid.

Aniline's oxidation product p-aminophenol did not give on the same diet any epithelial changes of this kind, which the aniline was shown to give even in half the dose.

Azotoluene gave earlier epithelial changes of the bladder, if the animals, before the application of the carcinogenic agent, had remained on the basic diet for 139 days.

The combination casein-riboflavine in test animals was shown to promote the excretion in the urine of ascorbic acid and of the oxidation products aminobenzoic acid and amino cresol both on feeding with azotoluene as well as with m-toluidine.

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*Fig. 7.*

Bladder changes, grade 1, after 258 days' feeding with 0.1 % m-toluidine, rat 1-2. $\times 144$.

*Fig. 8.*

Bladder changes, grade 3, after 265 days' feeding with 0.1 % o-toluidine, rat 3-3. $\times 40$.

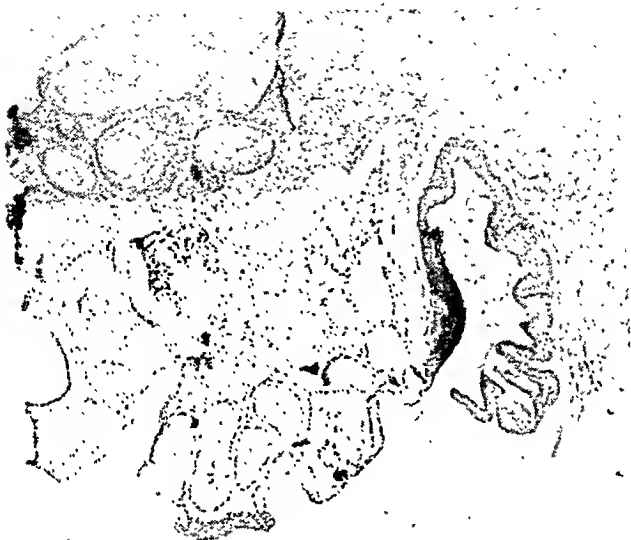


Fig. 9.
Bladder changes, grade 4, after 164 days' feeding with 0.1 % o-toluidine,
rat 2—4. $\times 40$.

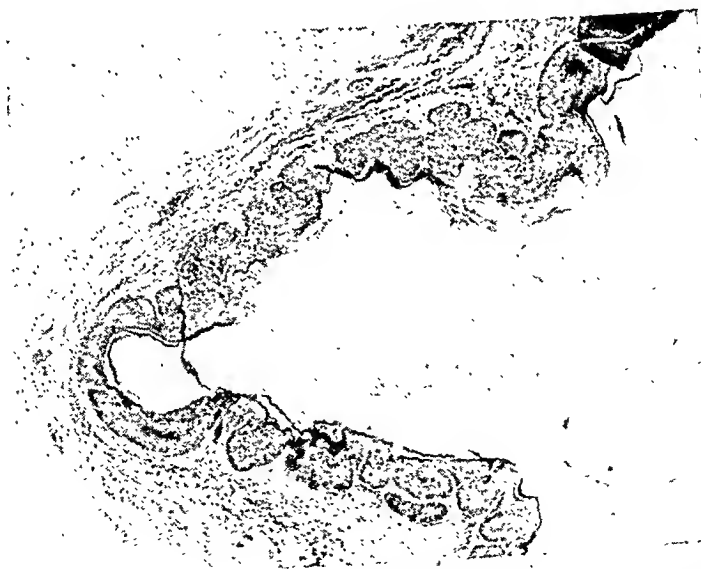


Fig. 10.
Bladder changes, grade 2, after feeding with 0.2 % m-aminobenzoic acid
during 262 days, rat 1—7. $\times 44$.

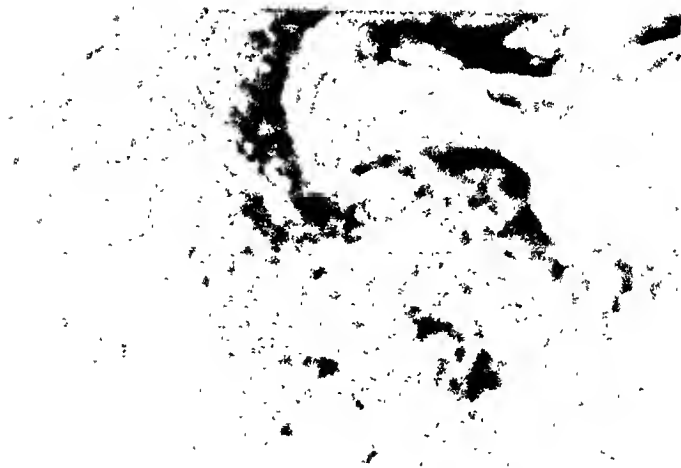
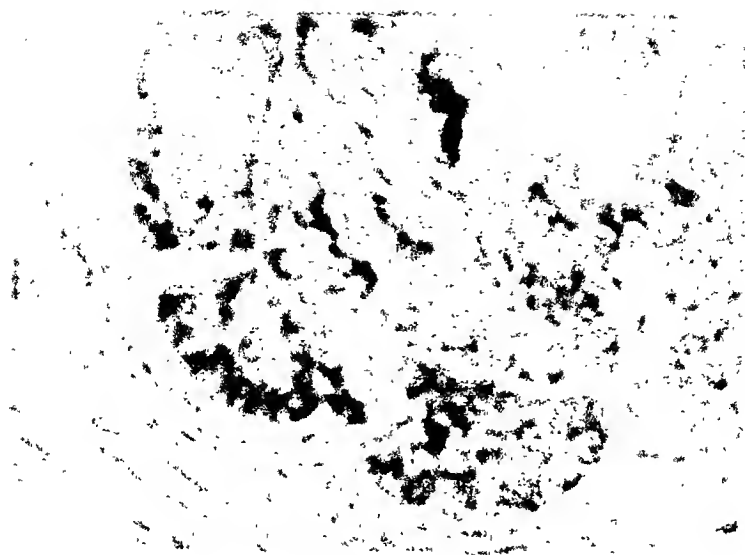




Fig. 13.

Changes of the renal pelvis, grade 1, after feeding with 0.2 % o-aminobenzoic acid during 176 days, rat 1-7. $\times 170$.



Fig. 14.

Bladder changes, grade 1, after 50 days' feeding with 0.3 % azotoluene, preceded by 139 days' basic diet without carcinogen, rat 1-35. $\times 170$.



Fig. 15.

Bladder changes, grade 2, after 45 days' feeding with 0.3% azotoluene, preceded by 139 days' basic diet without carcinogen, rat 1-36, $\times 170$.

THE EFFECT OF FEEDING OF ANILINE ON THE URINARY BLADDER IN RATS*)

By B. Ekman and J. P. Strömbeck.

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Ever since Rehn 1895 furnished his first report on tumours of the bladder as an occupational disease among workers in aniline factories an extensive literature has grown up dealing with these so-called aniline tumours. Considerable work has especially been devoted to finding out which substance is at work here. Occupational hygienic experiences from Germany point to aniline being really capable of procuring tumours, whereas this theory received no support in U. S. A. (Evans). In ordinary test animals aniline has generally shown itself to be inactive on the urinary bladder, apart from the appearance of hematuria, hyperemia and inflammation in the mucous membrane. Haemorrhagic cystitis has also been observed among workers handling aniline and its homologues (17). Among later investigators mention may be made of Berenblum and Bonser, who in 1937 reported successful feeding experiments with aniline. Kinoshita in 1940 considers that aniline is »not carcinogenic what soever«. It has even been proposed to substitute the classification aniline tumours by that of amino-tumours (2).

Occasional positive animal experiments with aniline have, however, been reported. Thus Perlmann and Staehler 1933 have briefly mentioned that they obtained fibro-epithelial tumours of the bladder in rabbits by subcutaneous injection of β -naphthylamine or aniline (1 mg. daily). They do not, however, furnish sufficient details. On the other hand, local application in the urinary bladder in rabbits of 1 % aniline water with a daily dose of 100 mg. has given rise to papillomatous tumours in the bladder vertex (21). It is, however, re-

*) This investigation was made possible owing to the financial support of the National Medical Research Council.

markable that the changes, which were not shown to be atypical, appeared already in the second to third week, and that there was no parallelism between the experiment period and the tumour development.

The occupational hygienic experiences concerning aniline as a cancerogenic substance thus diverge. Nor do the experimental animal investigations give clear results.

In previous investigations we have shown that simple decomposition products of azotoluene, o- and m-toluidine (5) and o- and m-aminobenzoic acid (6) are tumorigenic on a certain diet. These compounds could be considered as derivatives of aniline. Furthermore Strömbeck (19) has shown that 2,3'-azotoluene tumours in the bladder are only obtained on a rice diet relatively poor in vitamins and proteins, but, on the other hand, are not obtained on a complete diet. It can, therefore, be supposed that earlier investigators, who had received negative results in animal experiments, used a diet containing protective factors. It, therefore, appeared justifiable to test the cancerogenic qualities of aniline on a restricted diet.

In this connection it deserves to be pointed out that aniline in vitro is split off on the incubation of 2,4'-diaminoazobenzene, p-aminoazobenzene and p-dimethylaminoazobenzene with yeast (16). It is also obvious that aniline can be formed in vivo from p-dimethylaminoazobenzene; for, on supplying this substance p-aminophenol is excreted (18). Aniline, however, unlike the mother substance did not give any liver tumours (12).

Own investigations.

Albino rats of the laboratory strain, which had been employed in previous experiments, were fed, commencing on 25/1 1946, on 5—7.5 mg. (group I) aniline daily in a rice diet supplemented with about 10 g. of carrot a week. 20 males were used. About half of them died early, but for eleven the feeding period amounted to 144—256 days. The urinary bladder was cut in series (8 μ , each 25th. slice was stained). Some of them showed pronounced hyperaemia, even haemorrhages submucously and in the musculature. The epithelium, however, was everywhere normal without metaplasia, keratinisation or tendency to proliferation. Nor did some animals on a free diet and the same aniline dose show any epithelial lesions.

In June 1947 new experiments (group II) with the same strain of rats were begun. In these the animals (6 males and 6 females) received 0.10 % aniline to the synthetic basic diet (vitamin-free casein 12 %, glycerol 79 %, corn oil 5 %, salts and a restricted vitamin mixture*), according to the Madison group (11). The daily quantity of

* 0.2 mg. riboflavin, 0.3 mg. thiamine HCl, 0.7 mg. Ca-pantothenate, 0.2 mg. pyridoxine HCl, 3.0 mg. choline chloride per 100 g. diet.

food consumed amounted to 10—15 g. per animal, corresponding to 10—15 mg. aniline per day per animal. The initial weight lay between 160—215 g., an average of 185 g. All animals first increased in weight and reached their maximum after 27—110 days (73 days on a average) only to diminish to, on an average, 6 % beneath the initial weight at death, which occurred after 80—331 days (an average of 204 days).

The dead animals showed often pale organs. Sometimes the urinary bladder was expanded by a blood-coloured content. It contained bladder stones in three cases, all males. 5 animals were lost through cannibalism. On microscopic investigation of the urinary bladder (serial slice 10 μ , at least each 50th. being stained) among the seven cases examined there were found in 4 epithelial changes with metaplasia and epithelial proliferation. They have been graded in accordance with the previous paper (6) and were in each two cases of grade 2 and grade 3, respectively (Table 1 and Figs. 1—3). The epithelium of the bladder was often desquamated in places, the mucosa being the seat of granulation tissue permeated with leukocytes in the lesions. The changes are essentially the same as those observed when feeding rats on azotoluene (13, 19), toluidines (6, 19) and acetaminofluorence (7). Thus hyperkeratosis together with a more or less pronounced epithelial proliferation and even clear papilloma formation are found. Yet nowhere are signs to be seen of an infiltrative growth and malignity.

The agreement is also great with the tumours of the bladder obtained in rats on administration of oestronc (20) and diethylstilbestrol (3).

Another group (III) of rats of the same strain, 9 males and 1 female, likewise received aniline to 0.1 % in the diet during the entire experiment period until they died after 98—312 days (on an average 185 days) (see Table 2). During a first period, however, these animals had been used for metabolic experiments with determination of excretion products in the urine and during this period received varying additions to a basic diet, consisting of vitaminfree starch, salt and vitamin mixture. Immediately after this period they were placed in boxes in groups of 5 animals in each with the same dose of aniline and the same basic diet as in the previous series (group II). Thus the conditions of the experiment are not quite comparable. Yet three of the animals had epithelial lesions of the bladder (one of grade 1, two of grade 3) see Table 2.

The kidneys of four rats exhibiting bladder lesions grade 3, were examined microscopically. The renal pelvis showed in two animals (5, 02—7) epithelial changes corresponding to grade 2 (fig. 4). In two rats with a normal urinary bladder the renal pelvis was normal.

The basic diet used in group II has at the same time been fed to 12 rats of the same strain without an addition of aniline or any other carcinogen. In two of them appeared inconsiderable epithelial keratosis of, at most, grade 1. These must be connected with the one-sided

composition of the basic diet (6). The more pronounced lesions (grade 2-3), obtained by feeding with aniline on this synthetic diet, must obviously be ascribed to a tumorigenic effect of this substance. As was established in detail in the previous paper (6) the content of vitamin A in the diet used is sufficient for preventing vaginal keratosis and was estimated by analysis after the experiment's finishing of the fish-oil preparation employed to be at least 10 I. U. per day per rat. Estrogenic effect of aniline was not observed on investigation of the vaginal smears from 4 animals during a period of 13-18 days.

According to our opinion as previously advanced (5), the inhibiting effect of certain factors in a complete diet on the appearance of tumours of the bladder by feeding azotoluene to rats is dependent on an increased formation of oxidized metabolic products. Phenol is oxidized in the organism to p-aminophenol. This substance has therefore been given to rats on the same basic diet as group II. The experiment embraced 10 animals and p-aminophenol was given in the diet in 0.2 %. No epithelial lesions could be observed, in spite of the dose being double when compared with the aniline supplied.

Discussion.

Thus the investigations here described make it clear that aniline could give rise to tumours of the bladder, when the test animals receive a certain diet. They seem, therefore, to be of interest in conjunction with the investigations carried out against the background of the occupational hygienic significance of aniline.

Moreover, they can also be connected with our own previous investigations. On a certain diet deficient in vitamins and proteins the same kinds of lesions in the epithelium of the bladder are induced by azotoluene, o- and m-toluidine, o- and m-aminobenzoic acid and aniline. On the other hand, p-aminophenol is not tumorigenic. Aniline is the simplest compound in this series. Changes in o- and m-position to the aminogroup do not appear to play any part, whereas, on the other hand, an oxidation in p-position neutralizes the tumorigenic effect (p-aminophenol). According to this train of thought another of the decomposition products for azotoluene demonstrated by us, namely aminocresol, should not be tumorigenic; investigations on this substance are in progress. Furthermore, in conformity with the urinary output of aminocresol (5, 6) the excretion of p-aminophenol should be increased on the administration of diet factors which protect against the appearance of tumours. Studies on aniline metabolism with varying diets have, therefore, been initiated.

Table 1.
Bladder lesions in rats Group II following administration of aniline.

No.	Sex	Duration of experiment in days	Body weight (g)		Epithelial changes					Stones in the bladder
			Initial	Final	0	1	2	3	4	
1	M	142	210	170	+					—
2	M	267	215	245				+		+
3*)	M	167	185	150						
4	M	166	215	245	+					—
5	M	251	190	245				+		+
6	M	331	195	155	+					+
7*)	F	151	160	145						
8*)	F	138	165	165						
9	F	307	180	160			+			—
10	F	161	160	130			+			—
11*)	F	289	185	130						
12*)	F	80	160	145						

*) not examined.

Table 2.
Bladder lesions in rats Group III following administration of aniline.

No.	Sex	Duration of experiment in days		Body weight (g)		Epithelial changes					Stones in the bladder
		Period I	Period II	Initial	Final	0	1	2	3	4	
02—1	F	224	88	153	145		+				—
02—2*)	M	224	25	145	260						
02—3*)	M	67	43	110	280						
02—4	M	53	127	150	240	+					—
02—5	M	224	73	155	200	+					—
02—6*)	M	46	52	115	185						
02—7	M	71	101	180	170				+		—
02—8	M	111	68	170	120	+					—
02—9	M	73	55	200	175						—
02—10	M	74	49	200	175				+		+

*) not examined.

Summary.

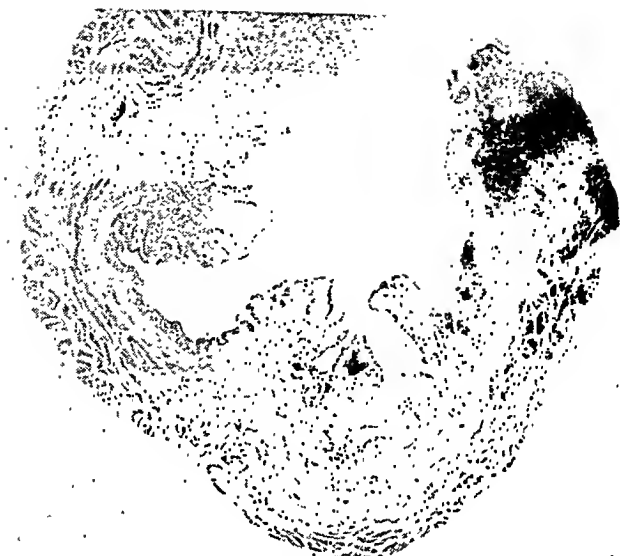
By administration of 0.1 % aniline in a basic diet consisting of casein, corn oil and glycose with a restricted vitamin content to a not systematically inbred laboratory strain of white rats, which for many years had been constant, keratosis and proliferation of the bladder

epithelium up to papilloma formation, though not malignant, were obtained. P-aminophenol in a double dose did not give such lesions.

Oestrogenic effect was not obtained by the aniline dose used in the diet under discussion.

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*Fig. 1.*

Bladder lesions, grade 3, after 267 days feeding with 0.1 % aniline, rat 1. The epithelium partially ulcerated with granulation tissue in the defect. $\times 17$.

*Fig. 2.*

Bladder lesions, grade 3, after feeding with 0.1 % aniline during 251 days, rat 5. $\times 44$.

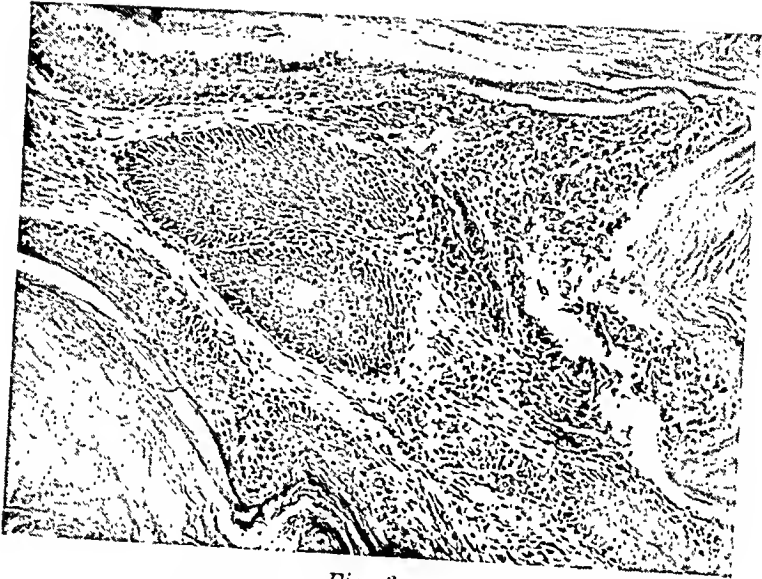


Fig. 3.
Bladder lesions, grade 3, rat 5. $\times 170$.



Fig. 4.
Epithelial lesions of the renal pelvis, rat 5 (the same animal
as in figg. 2 and 3). $\times 125$.

was changed to brother-sister matings. Only a part, therefore, of the later material was from this more highly inbred strain. In this latter series (23) a still high frequency of tumours of the bladder (44 % tumours of the bladder and 7 % renal tumours in 108 animals — roughly as many males as females) is obtained. *Cantarow* (1946) uses a Sherman strain, which obviously has a very low frequency of tumours of the bladder.

The importance of strain-differences in varying tendencies to bladder-tumours through carcinogens other than AAF has only been demonstrated, as far as is at present known, by *Dunning* et al. (6). By implanting pellets containing diethylstilbestrol, papilloma of the bladder was obtained in the majority of males of the Copenhagen line and to a less degree in the females. In AXC-line tumours of the bladder developed with this method to a still less degree and not at all in a third line (*Fischer*), which was also used with negative result concerning tumours of the bladder in the experiments with AAF described above. Whether diethylstilbestrol is active as a carcinogenic agent or only — like the estrogenic substances with experimental mammary cancer — has a provocative importance, constitutional factors seem here also to play a part in the development of tumours of the bladder.

Harris, in America, who used the same strain as *Bielschowsky* in England and like the latter obtained few tumours of the bladder or none at all, considers »that the incidence of tumours to be expected in the various tissues is determined by the strains of the rat«.

Peacock (1944) discusses »the relative values of stock mice and inbred lines for investigating the competence of tissues to react on carcinogens. In investigating a problem that is primarily concerned with human pathology it is well to bear in mind that there are no inbred lines in human beings, and that experiments carried out in purebred animals may have a very limited bearing on the etiology of malignant neoplastic diseases in corresponding sites in human beings«. If inbred lines be not used, the animal stock employed must be kept as pure as possible.

The constitutional factor obviously plays a leading part and must be taken into consideration in experiments aiming at producing tumours of the bladder with AAF. This is certainly the explanation of many of the varying results in the literature.

Ad. 2. The importance of different diet factors is nowadays well known in connection with azo compounds producing tumours of the liver; p-dimethyl-aminoazobenzene, o-aminoazotoluene, etc. That such factors are also of importance in the development of tumours of the bladder through azotoluene has been shown by *Strömbeck* (20, 21). The strong carcinogenic effect in AAF seems perhaps a priori to be less easily influenced by diet factors. *Wilson* et al. (23) complemented their diet by the addition of cod liver oil, brewers' yeast and wheat

germ, using a dose of 0.125 % AAF in 41 days, after which 0.031 % until 143 days. On the 174th day 4 animals had external tumours and postmortem examination revealed »possible thickening in the bladder«. They consider that »there was no indication that the dietary supplements had modified the types of lesion, but report that »another study still in progress suggests that the diet may be of great importance in regard to the carcinogenicity of AAF«.

Harris and Bradsher (1946) have studied the effect of dietary modifications on the development of hepatic tumours by AAF. Neither a diet rich in casein-riboflavine nor additions of liver extract, which in the same strain of rats retarded the growth of tumours in experiments with p-dimethylaminoazobenzene (butter yellow), gave any significant difference in hepatic tumour development, when 0.04 % AAF was added to the respective diets. *Harris* (1947) in a later series did not obtain any protective effect with synthetic diet and the same dose of AAF, and considers that this indicates that the mechanism of tumour formation by the fluorene derivatives differs from that concerned with p-dimethylaminoazobenzene-carcinogenesis.

In view of the strong carcinogenicity in AAF it seems, however, necessary that the effect of diet factors should be tested in lower doses of the carcinogen than these authors have employed. Experiments arranged in conformity with this principle were initiated in 1945.

OWN INVESTIGATIONS

Series A.

Of 4 groups of white female rats, each comprising 5 animals and of an average weight of 178—196 g., two groups were fed on a rice diet with 10 g. of carrot each week with an addition to this of 0.031 % AAF (5 animals) and 0.016 % AAF (5 animals). The remaining two groups received a complete diet (*Hammarsten* (11) 211, containing casein, rice flour, sugar, arachis oil, cod liver oil, yeast, wheat sprouts and salt mixture), which had previously shown itself capable of protecting the rat-strain employed from the formation of azotoluene tumours in the bladder (21). One of these two latter groups received an addition to the diet of 0.031 % AAF, the other an addition of 0.016 % AAF. All were fed ad libitum and were killed after 162 days (one, however, died after 158 days).

The result was negative to such a degree that not one of the 20 animals showed tumours of the bladder. On the other hand, *macroscopic lesions of the liver* were found in both groups, which had received 0.031 % AAF. These were substantially more pronounced in

the group which had been on a complete diet (see Fig. 1). The liver weights were both absolutely (average 17.1 and 7.5 g. respectively) as well as in percentage of the body weight (8.5 and 6.9 % respectively) greatest in the latter compared with the animals on rice diet. In the majority of these 10 animals with the higher AAF-dose proliferative lesions of gall ducts and liver cells were microscopically found, but most pronounced in animals with the 211-diet.

In both groups with the lower AAF-dose (0.016 %) macroscopic lesions of the liver were absent in all 5 animals on the 211-diet. In the 5 on rice diet the liver was macroscopically normal in 2 animals but revealed slight macroscopic lesions in 3. Only these 3 had microscopic lesions in the liver with an increase in connective tissue, proliferation of the gall ducts, and in one rat incipient adenoma formation as well.

These findings of our investigation seem to speak in favour of the theory that there may be factors in the 211-diet, which retard the development of liver lesions when a sufficiently low (0.016 %) supply of AAF has been administered. With larger doses (0.031 %), however, of AAF the growth occurs more quickly on 211-diet than on a rice diet.

Series B.

8 parabiotic pairs (litter-mate males) of Täby strain (not the previously used strain), in which double-sided nephrectomy had been carried out on the left partner 25—39 days after establishment of the parabiosis, received 0.031 % AAF in 211-diet ad libitum from 1—2 weeks after the nephrectomy during 73—197 days. They died after 139—354 days of parabiotic life. Tumours of the bladder were absent in both the left (kidneyless) as well as the right partner in the 8 pairs. Thus the experiment, intended to obtain in this manner proof for the urogenic or haematogenic theory, failed. The left (kidneyless) partner was consistently atrophic and hyperaemic, and showed pronounced sclerosis of the aorta, whereas the right one was fat and pale. *Liver lesions* were found in all 8 right animals and were consistently most pronounced in them (see Fig. 2). They were missing in at least 3 of the left animals.

The metabolic conditions are very complicated in the parabiotic animals. One would, however, be justified in considering that the better nutrition in the right animal favoured the development of liver tumours.

Series C.

In investigations on the influence of butter yellow in forming liver tumours the Madison group (11) made use of a synthetic diet containing corn oil and a restricted quantity of vitamins of the B-group.

The same diet was used by us in investigations to determine the capacity of azotoluene and its split products, and of aniline to produce tumours of the bladder (*Ekman — Strömbeck* 9, 10). Parallel with this and roughly at the same time experiments were initiated in 1947 with AAF*) added to the Madison diet. In accordance with information from the literature to which reference has previously been made and according to our own investigations a tumour-protective effect in riboflavine-casein and possibly ascorbic acid may be expected. The Madison diet contains a certain quantity of casein (12 %), and with the object of testing a protective effect in riboflavine and ascorbic acid 3 experimental series were set up in the following manner:

Each group comprised 12 rats with an average weight of 130—140 g. (6 males, 6 females) and all the groups received an addition of 0.015 % AAF. The animals consumed on an average 10—15 g. diet per day.

Group I received the Madison group's basic diet: 79 % glucose, 12 % vitamin-free casein, 5 % corn oil, 4 % salt mixture and per 100 g. diet 0.2 mg. riboflavine, 0.3 mg. thiamine, 0.7 mg. Ca pantothenate, 0.20 mg. pyridoxine hydrochloride, and 3.0 mg. choline chloride. Added to this 1 drop of *Oleum jecoris forte* or *Guttae adevitami**) per rat each month.

With the preparations used the supply of vitamin A, calculated daily, amounted to at least 10—40 I. U. per day.

Group II received 2.0 mg. riboflavine per 100 g. diet but otherwise the same diet as group I.

Group III received the same diet as group I but daily received 20 mg. ascorbic acid in subcutaneous injection.

By reason of intercurrent infections and cannibalism not all animals could be examined. The average spontaneous length of life amounted to 208, 156 and 190 days in the respective groups. All animals surviving for 300 days (2 in each of groups I and II, and 4 in group III) on the carcinogenic diet, which they received daily all the time, were killed. Bladder stones were found in 3 animals in group I, not at all in any of group II, and in 1 animal in group III.

Epithelial changes of the bladder of varying degree were found in 20 of 25 animals examined, roughly equally distributed in both sexes and in the 3 groups (7/10, 5/5, 8/10). Cases of advanced changes were

*) The preparation was kindly made up by Mr. K. G. Rosdahl of A/B Ferosan, Malmö. For the synthesis fluorene was used, placed at our disposal by the generosity of Koppers Company, Pittsburg.

*) At a control analysis in A/B Leo, Hälsingborg in August 1948 a small remainder of *oleum jecoris forte*, bought 4. XI. 1947, was shown to contain 25000 I. U. vitamin A per g., and *Guttae adevitami*, bought 3. 3. 48, 6500 I. U. vitamin A per g. The average weight of a drop of the preparations correspond to 50 mg.

found in all 3 groups. Cancers did not develop. Transplantation experiments from pronounced cases are in progress.

Papillomatosis could be observed with loupe only in the most extreme cases. All bladders (10,5 and 10 in the respective groups) collected were cut in serial sections (10 μ , at least each 50th. stained). The microscopic lesions agreed with those described by previous authors (22) and have been schematically divided up into four grades:

- 1) Thickening of the epithelium and metaplasia with keratinization but without signs of proliferation. Most often it has been a question of locally limited lesions (see Fig. 3).
- 2) Epithelial metaplasia with keratinization — usually diffusely spread in the bladder — with proliferation most often in the form of epithelial infolding and down growths to the submucosa. Also smaller epithelial pearls with cornification (see Fig. 4).
- 3) More pronounced proliferation with a tendency to keratotic papillomas, and often large cyst formations (see Figs. 5—6).
- 4) More pronounced lesions, possibly in some places with atypical epithelium (see Figs. 7—8).

The kidneys of three rats presenting bladder changes (I—4, I—5, III—9) were examined microscopically. Epithelial changes of the renal pelvis were missing. Same was the finding in two rats (I—2, III—10) with normal bladders. An unique case can be reported of diffuse metaplasia with cornification but without proliferation of the epithelium of the renal pelvis by normal bladder. (I—7).

For the reasons put forward in a previous paper (*Ekman and Strömbeck* (9)) the lesions of grade 1 could not be definitely ascribed to the carcinogen employed.

As epithelial lesions of the bladder similar to those obtained here have also been demonstrated after administration of estrone (16) and diethylstilbestrol (6), the possibility of an estrogenic effect of AAF has also been tested. In 4 rats, which during several months were kept on the same diet as group I in series C with administration of 0.015 % AAF, a generally normal series of vaginal smears has been obtained during a period of 13—18 days. Thus no estrogenic effect could be demonstrated of AAF in the dosage used.

Macroscopic lesions of the liver (cirrhosis, hepatomas) were present in about one half of all cases. They were more common in animals presenting epithelial bladder changes than in animals with normal bladder epithelium.

Discussion.

Acetaminofluorene has given *liver changes* in all 3 series (A, B, C) of the material. In series A (where the animals did not get bladder changes) liver changes developed on a dosage of 0.016 % and an experiment period of 162 days only on rice diet but not on a complete diet. On a dosage of 0.031 % they developed within 162 days both when rice diet was used as well as on a 211-diet, and were more advanced on the complete diet. In series B (parabiosis), in which bladder changes did not occur either, the liver changes in the hypertrophic right animal were more usual and more advanced than in the left partner and were macroscopically visible already after 73 to 157 days. In series C (Madison diet and low AAF-dosage = 0.015 %) the liver tumours were somewhat less usual but seemed, in spite of the experiment period being longer in a number of cases, not to attain such a large quantitative development.

The 3 series differ in regard to the occurrence of *bladder changes*. In the first two these are completely missing whereas in the third they appear with great regularity. The parabiotic animals, bought from an animal dealer in another place, must in this connection be left out of account. Series A and C, on the other hand, consist of rats from a strain, originally emanating from Copenhagen, which has been bred for some decades at the medico-chemical institute. Fresh breeding-stock has not been brought from outside during this period. Spontaneous tumours have been seldom observed during the latest ten-year period. The animals in series A were born in Dec. 1944, those in material C in June, 1947. Simultaneously with these series experiments were carried out with azotoluene and m- and o-toluidine, which during both periods gave positive results concerning tumours of the bladder. Thus no essential difference in the animals' constitutional tendency to bladder tumours would appear to exist between the A and C series.

Dosage, experiment period and dietary distribution are not entirely alike in two groups now compared. In series A (in which bladder tumours did not develop) 10 animals (all females) received 0.031 % AAF during 158—162 days (on an average 161.6) and 10 others (also females) 0.016 % AAF during 162 days. Thus the dosage for half of the animals is greater than in series C, in which tumours of the bladder were formed. There, instead, the experiment period is usually longer. Only 9 animals in material C have died during the period of 150—200 days. The average for them is 168.9 days. In these 9, however, bladder lesions were found in 7 animals:

of grade 1 in 4 cases, after 169, 169, 177, 188 days	
- - 2 - 2 - - 156, 165	-
- - 3 - 1 case - 166	-
not at all - 2 cases - 165, 165	-

Thus, in spite of giving twice as big a dose of AAF to half the number of animals as to the animals on Madison diet in series C, bladder tumours have not developed in the rats on 211 and rice diet in series A. The experiment period was about the same in both series.

Wilson et al. (23) found that the animals receiving AAF for 75 days or more developed their tumours in »about equal times« (227—295 d.). They observed carcinoma of the bladder in 3 animals already after 194 d.

The age is somewhat higher in series A (average weight 184 g.) than in series C (average weight 135 g.). This difference ought not to be of any great importance. Wilson (24) has found the same results in 440—612 days old animals, as in the animals where the feeding with AAF began »shortly after weaning«.

In his first publication Wilson (22) stated that the toxicity of AAF was greater for males, for which reason solely females were used in series A. In series C, where as many males as females were used, no difference is found in the incidence of epithelial changes in males and females. Wilson (23) also found that in 108 animals (53 males, 55 females), which developed bladder tumours in 44 % and renal tumours in 7 % »the incidence and time of development of these two types of lesions were about the same in both sexes«. Thus the essential difference in result between series A and C should depend on the diet employed.

If a comparison is made of the diets used, of which only the Madison diet gave tumours of the bladder, it is found that the supply of carbohydrate is covered in the Madison diet by glucose, and in both the other diets essentially by rice flour. The supply of carbohydrates in all diets is sufficient from the calorific point of view, and there is no reason to suppose that the kind of carbohydrate administered should play any part in the incidence of tumours. The glucose of the Madison diet has the definite advantage that we have in it a uniform substance without a varying mixture of unknown diet factors of, for instance, the vitamin type.

The supply of protein in the Madison and 211 diets occurs through casein, and in the case of the latter through the protein content in rice flour. The protein content in the Madison diet amounts to 12 %, which lies considerably beneath optimum for growth (roughly 18 %), and in the 211 diet to 19.7 %. In the rice diet the protein content is only 6.7 %. There the protein is entirely of vegetable type.

In the question of fat, there also exist fundamental differences. The addition of fat in the Madison diet is entirely satisfied by corn oil which amounts to 5 %. The 211 diet contains partly fat, found in the rice flour, and partly 3.7 % arachis oil. The total quantity amounts to about 5.4 %. The rice diet contains ca. 2.5 % fat, which derives from rice flour.

211 and Madison diets contain both comparable and adequate salt mixtures. Addition of salt is not included in the rice diet.

Comparisons between the vitamin content of the different diets are very difficult, as only the Madison diet contains fully defined ingredients.

The vitamin B content is fully adequate in the 211 diet through a rich addition of yeast, which is conditioned in the rice diet by the rice flour's probably changing content of group B vitamins and in the Madison diet by the addition of pure vitamin preparations. Whether it be in the rice diet or the Madison diet the content of vitamin B is insufficient for normal growth.

The supply of vitamin A is satisfactory in all diets from a quantitative point of view: yet there is the difference that the supply occurs only once a month in the Madison regime, but in the other diets it is more continual. This may possibly be of importance. The rat's ability to absorb and store occasional doses of vitamin A is considered limited (12). A low content of vitamin A also promotes the development of bladder tumours through oestrone (16).

The diet ingredients, which can be placed in connection with the varying incidence of tumours, are evidently fat, protein and vitamins.

It seems to be of considerable importance that the Madison diet, which alone gives tumours of the bladder, is also the only diet to contain corn oil. As regards protein the Madison diet takes up an intermediary position; it has a higher protein percentage than the azotoluene tumour-promoting rice-diet, and a lower protein percentage than the azotoluene tumour-protective 211 diet. Concerning the vitamin supply it is seen from the investigation of vaginal smears described above and from the control of the vitamin preparations carried out afterwards that there has been no deficit of vitamin A in the animals on the Madison diet. Group II in series C received large quantities of riboflavine as addition to the Madison diet without any effect on the incidence of tumours. According to our previous investigations riboflavine, in order to exercise its full effect, ought to be combined with casein, and the casein content is relatively low in the Madison diet. On the other hand, tumours are absent in the rice diet group in which the riboflavine content is probably low and casein is entirely wanting.

Thus of the diet factors, which are at present accessible for analysis, it would appear that a certain importance should above all be ascribed to corn oil as concerns the differences in tumour incidence already observed. Corn oil, according to the Madison group's investigations, seems to have a direct tumour-promoting effect, when it is a question of the development of liver tumours after administration of p-dimethylaminoazobenzene. Thus if corn oil was substituted for mineral oil no tumours developed. If the content of corn oil was raised from 5 % to 10 % a higher incidence of hepatoma was obtained. Our

experiments with AAF would appear to show that corn oil in the rat strain used is of no importance for the development of liver tumours but may well be for tumours of the bladder.

Summary.

It has been possible to induce epithelial changes of the bladder (keratosis, papillomatosis) in a constant, but not pure, laboratory strain of albino rats by feeding with 0.015 % acetaminofluorene in a synthetic diet with a low protein (casein) content, containing corn oil and of relatively scanty vitamin content. Animals from the same strain had not developed lesions of the bladder, when the acetaminofluorene was added in the same or larger doses to a complete diet (Hammarsten's 211 diet) or rice diet with carrots added. Thus, in addition to the differences of strain mentioned in the literature, the differences in diet would seem to explain the different tendencies to tumours of the bladder on feeding with AAF.

Lesions of the liver were obtained in the majority of the animals in all experimental series. Here diet also seems to play a certain part in their appearance and development. Acetaminofluorene has not shown any estrogenic qualities in the dosage used here.

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Table 1.
Bladder lesions in rats fed 0.015 % acetaminofluoren. Series C.

Nr.	Group I.													Group II.													Group III.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
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	1	2	3	4	Final	0	1	2	3	4		Stone	0	1	2	3	4	Final	0	1	2		3	4	Stone	0	1	2	3	4	Final	0		1	2	3	4	Stone																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
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* Not examined.

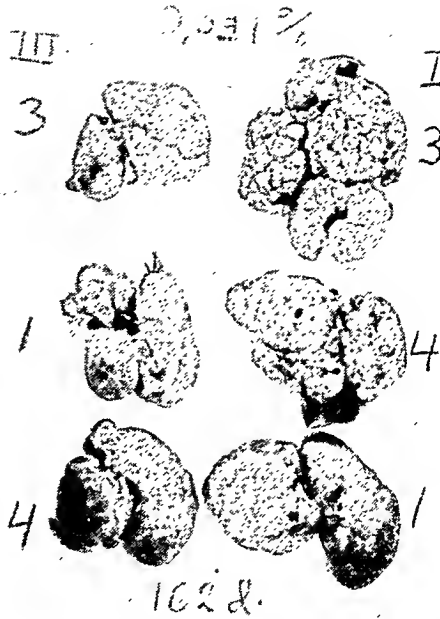


Fig. 1.

The liver in rats, which have received 0.031 % acetaminofluorene in a rice diet (left column) and in a complete diet (right column), all during 162 days.

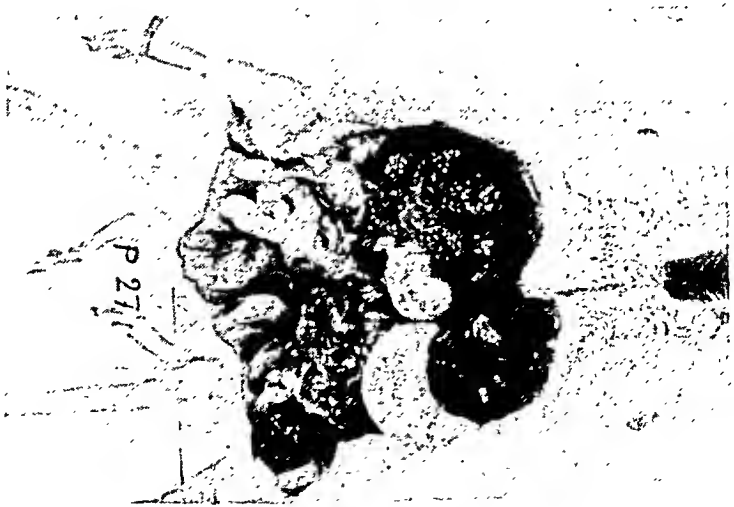


Fig. 2.

Parabioc animals, which have received 0.031 % acetaminofluorene in a complete diet during 122 days after the left partner has undergone bilateral nephrectomy. Liver lesions greater in right-hand animal.



Fig. 3.

Bladder lesions, grade 1, after 309 days' feeding on 0.015 % acetaminofluorene, rat 2-5. $\times 170$.

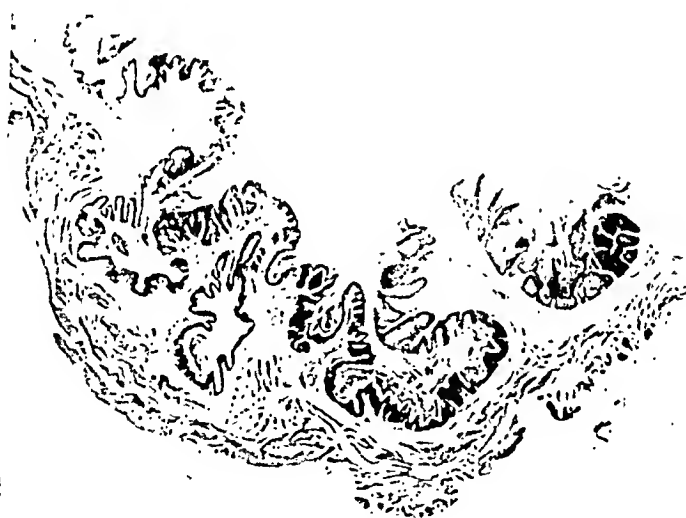


Fig. 4.

Bladder lesions, grade 2, after 309 days' feeding on 0.015 % acetaminofluorene, rat III-7. $\times 17$.

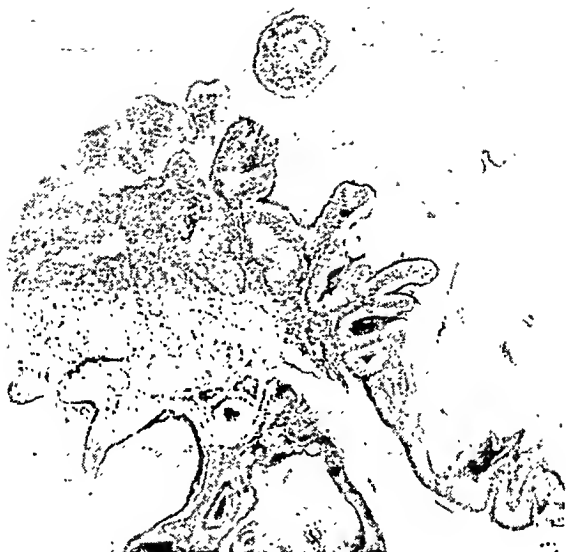


Fig. 5.

Bladder lesions, grade 3, after 309 days' feeding on 0.015 % acetaminofluorene, rat I-10. $\times 44$.



Fig. 6.

Bladder lesions, grade 3, after 273 days' feeding on 0.015 % acetaminofluorene, rat I-11. $\times 170$.



Fig. 7.

Bladder lesions, grade 4, after 308 days' feeding on 0.015 % acetaminofluorene, rat II-11. $\times 44$.



Fig. 8.

Bladder lesions, grade 4, after 304 days' feeding on 0.015 % acetaminofluorene, rat III-9. $\times 44$.

HISTOLOGICAL INVESTIGATION OF THE RELATIVE FLUORESCENCE OF 3.4 BENZPYRENE, TAR, AND 9.10-DIMETHYL-1.2-BENZANTHRACENE IN RABBIT-SKIN

By C. G. Ahlström and N. B. Berg.

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Earlier investigations have shown that when 3.4-benzpyrene is applied to the skin of a mouse, the carcinogenic agent or its fluorescent derivatives will fluoresce in the shape of perinuclear rings due possibly to an accumulation of the agent in the mitochondria of the epidermal cells. This annular type of distribution has been observed in the cells of the epidermis of the interscapular region but not in the skin of the ears, paws, or tail, where the reaction to benzpyrene differs also in other respects (Ahlström & Berg).

The purpose of the present investigation was to attempt to ascertain whether the distribution of the fluorescence in rabbit-skin painted with benzpyrene would be similar to that in the skin of the mouse. Another purpose was to compare the distribution of the fluorescence of 3.4-benzpyrene, tar, and 9.10-dimethyl-1.2-benzanthracene. As 3.4-benzpyrene is but feebly carcinogenic in rabbit-skin and as tar and especially 9.10-dimethyl-1.2-benzanthracene have a relatively strong carcinogenic potency, it was believed that a study of the relative fluorescence of these three agents might be of interest.

Methods and Material.

The material consisted of white rabbits weighing 2000—3000 gms. Acetone solutions containing 0.5 per cent 3.4-benzpyrene, and 9.10-dimethyl-1.2-benzanthracene, respectively, were used. The 3.4-benzpyrene was manufactured by Hoffman la Roche, the 9.10-dimethyl-1.2-benzanthracene by Prof. H. Lund, Aarhus, Denmark, and kindly supplied by Prof. J. Engelbreth-Holm. The tar was a horizontal retort

tar from Leeds, and was kindly placed at our disposal in 1937 by Prof. R. D. Passey.

The carcinogenic agents were applied to the inner surface of the ear and to the clipped back of the animals. In several of the experiments one and the same rabbit was painted with two carcinogens, for example, tar on the inner surface of one ear and the corresponding side of the back, and benzpyrene on the other ear and flank. Biopsies were punched from the ear with the aid of a trephine, and excised from the painted dorsal areas, with a knife. The specimens were fixed in a 10 per cent solution of formalin, after which frozen sections 10 microns in thickness were cut on the freezing microtome. Unstained sections were then studied under a Reichert Lux UV fluorescence microscope. Other parts of the excised specimens were fixed in Regaud's fluid. In addition to routine staining with eosin, Regaud's and Bensley's mitochondrial staining methods were also employed. In contradistinction to earlier experience on mice, when Bensley's method produced the best results, Regaud's method now proved the better of the two for rabbits.

Observations.

Fluorescence of normal rabbit-skin. The fluorescence of normal rabbit-skin essentially resembled that observable in normal mouse-skin. The epidermis had a dirty greyish-blue or greyish-green fluorescence, in which the cell nuclei were sometimes recognized in the form of optically empty spaces. The keratin and the hairs fluoresced faintly yellow. The dermis was of a faint, dirty greyish colour, whereas the elastic fibers were apparent as rather distinct, yellow, twisted streaks. The elastic layers in the larger blood vessels fluoresced yellowish-white.

Reactions observed in rabbit-skin after the application of 3.4-benzpyrene.

The fluorescence of the skin after a single application of benzpyrene was investigated on 7 rabbits. Biopsies were taken $\frac{1}{2}$, 2, 4, 8, 19, 24, 36, 48, 60, 72, and 96 hours after the application. Three of the rabbits were re-painted on the previously painted areas of the skin a few minutes before death. Six rabbits were painted 4—5 times at intervals of 4 days. In addition hereto tar and benzpyrene, respectively, were applied to the opposite ear and flank of 4 other rabbits.

As was the case in earlier experiments performed on mice, the benzpyrene was deposited on to the surface of the skin in the form of intensely fluorescent yellow or yellowish-green needle-like crystals. The keratin layer and the contents of the sebaceous glands fluoresced

brilliantly bluish-white. The fat droplets in the sebaceous glands gave the cytoplasm a granular appearance, whereas the cell nuclei appeared as dark spots devoid of fluorescence.

The benzpyrene rapidly entered the epidermal cells. A few hours after the application of the carcinogen the cytoplasm of the cells in the stratum basale and spinosum exhibited a rather strong violet or bluish-violet fluorescence, which was diffusely distributed within the cytoplasm. It was not stronger in the perinuclear area than in the peripheral zone. The cell nuclei were apparent in the shape of round or oval empty spaces containing no fluorescent substance. The inter-



Fig. 1.

Pale, fluorescent rings around the nuclei within the stratum basale and spinosum 3 days after a single application of 3,4-benzpyrene.

cellular bridges exhibited a rather marked whitish fluorescence, which gradually merged into that of the keratin layer. The fluorescence remained unchanged for the first 24 hours after the application of the carcinogenic agent. No difference was detectable between the fluorescence of the skin of the ears and that of the back. The connective tissue in the corium displayed a bluish-violet fluorescence, whereas that of the ear cartilage was brighter and sometimes whitish.

After 24—48 hours the fluorescence began gradually to fade. At the same time, however, a difference was detected between the outer and inner zones of the cytoplasm, the fluorescence in the latter zone becoming slightly brighter than in the former (Fig. 1).

Already at the 24 hour-stage the nuclear membrane was sometimes invested with a thin coating of condense dfluorescent material, which

almost seemed to impregnate the actual nuclear membrane. Usually, however, the perinuclear accumulation of the fluorescent substance was not visible until the second or third day after application. The perinuclear rings were by no means so distinct as in the skin of mice painted with benzpyrene, and were visible only in scattered cells within the painted areas of the skin. No fluorescent granules were distinguishable in the cytoplasm. After four days, only the keratin layers of the skin fluoresced. The ear and the back exhibited no difference with respect to the intracytoplasmic distribution or persistence of the fluorescence.

In the majority of cases an additional application of benzpyrene to the clipped areas immediately before the animal was killed evoked more conspicuous perinuclear rings. In some parts of the re-painted areas there appeared a strong perinuclear fluorescence, which was sometimes brighter around the basal poles of the cell nuclei. The distribution of the fluorescence was essentially similar to that observed in earlier experiments on mice, although it should be definitely pointed out that even after the repeated application of the carcinogenic agent the perinuclear arrangement in the rabbit was never distinct. The perinuclear rings manifest in rabbit-skin were hazy and faint, and often observed only in a few scattered cells within the painted areas.

Observations made on tarred rabbit-skin.

Six rabbits were tarred 1—5 times and the fluorescence of the skin then studied. Biopsies were taken 1, 2, 3, 4, 6, and 8 days after the first application of the carcinogenic agent. When the tar was applied several times to the same areas, biopsies were taken 24 hours after the last tarring.

In the fluorescence microscope the tarred skin gave a very colourful picture difficult to describe. The actual tar had the appearance of a dark-brown or black crust on the surface of the skin. The underlying keratin layers as well as the hairs and the sebaceous glands fluoresced bright yellow or yellowish-white. The fat droplets in the sebaceous gland cells were apparent in the form of distinct granules. The epidermis was rather bright greyish-blue or greenish-blue and differed markedly from the bluish-violet colour of the skin treated with benzpyrene. The intercellular bridges were white and distinctly separated the individual cells in the stratum spinosum and the stratum granulosum. The cell nuclei were apparent as optically empty spaces. About 24—48 hours after the application of the tar the fluorescence around the nuclei began to increase in intensity. At the 24 hour stage the nuclei were surrounded by thin fluorescent rings closely encircling the nuclear membrane. About 48 hours after the application, however, the nuclei in the stratum basale, spinosum and granulosum were regularly enclosed by rather thick fluorescent rings, which were as a

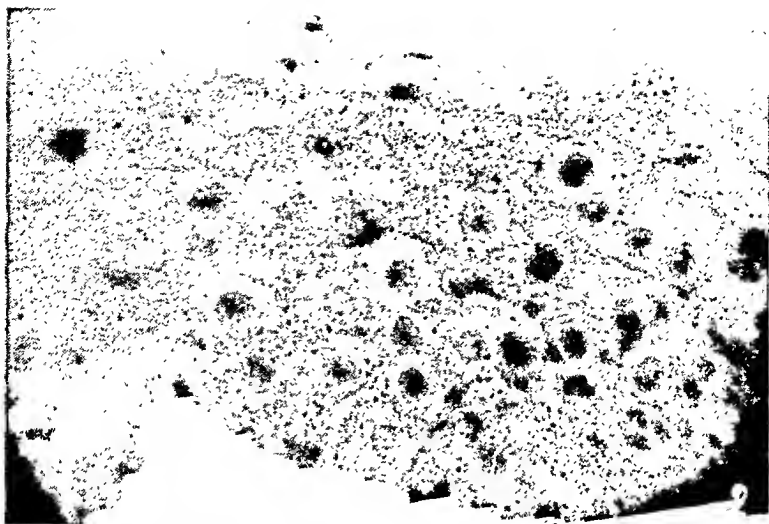


Fig. 2.

Bright and thick fluorescent rings around the nuclei within the stratum spinosum 48 hrs. after tarring.

rule most distinct in the basal cell layers (Fig. 2). In addition hereto, the cytoplasm often contained small, rather bright yellowish-white fluorescent granules, localized in the immediate vicinity of the nuclei, often accumulated around the outer and inner nuclear poles (Fig. 3). In the course of the following days the fluorescence gradually faded and assumed a bluish-violet shade. On the 8th day after application

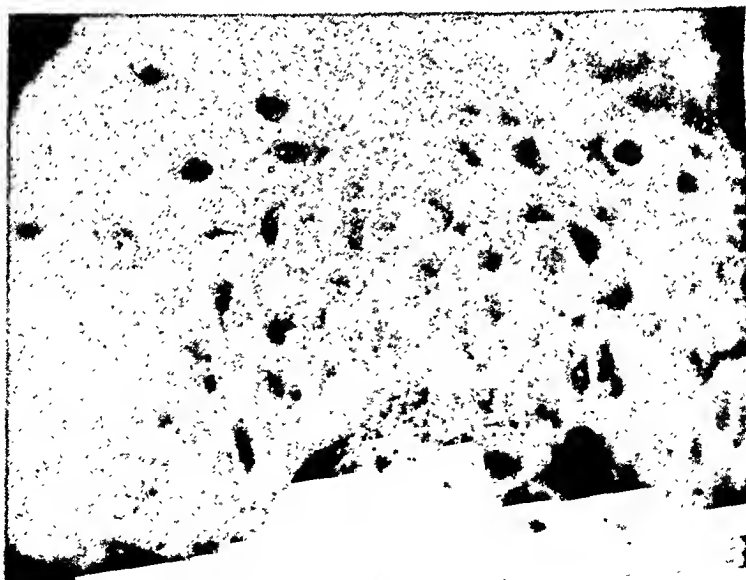


Fig. 3.

Perinuclear rings and fluorescent granules in the cytoplasm 72 hrs. after the tarring.

of the tar the perinuclear rings, though faint, were still observable, and the fluorescence of the epithelium was only pale bluish-violet.

Repeated applications of tar gave a more impressive fluorescent image. The epithelium fluoresced bright gray-blue-green and the cells within the stratum spinosum were circumscribed by thick, conspicuous greyish-blue rings. They were somewhat less pronounced around the cells within the stratum basale. In addition hereto, the cytoplasm, especially within the stratum basale, displayed numerous perinuclear accumulations of fine granules with distinct bluish-white fluorescence. With the continued application of tar these granules disappeared or became considerably scantier. The fluorescence of the perinuclear rings was not more intensive after three or four treatments than it was after two.

No difference was observed between the fluorescent image of the skin of the ear, and that of the back.

Observations made on rabbit-skin after the application of 9.10-dimethyl-1.2-benzanthracene. Compared with tar and benzpyrene, the fluorescence produced by 9.10-dimethyl-1.2-benzanthracene was not only weak but it also faded on exposure to ultra violet rays. About 12—24 after its application the horny layers and the sebaceous cells fluoresced rather intensely bluish-white. The fluorescence of the epidermis was bluish-violet, but was however so dark that it was difficult to distinguish the individual nuclei, which were apparent as round empty spots. The corium manifested its natural dirty greyish-green fluorescence; only the fibers were violet. On account of the weak fluorescence, within the epidermis it was difficult to analysis its distribution inside the individual cells. However, about 24 hours after the application of the chemical to the surface of the skin, scattered fluorescent crescents or rings began to appear within some of epithelial cells (Fig. 4) in a few of which fluorescent granules were also observed. On the third day after painting the fluorescence in the epidermis was so faint that details could no longer be distinguished.

Analysis of stained specimens from treated areas of the skin.

The main purpose of the histological analysis of stained specimens was, as also in our earlier investigations on mice, to endeavour to investigate the substrate of the intra-cytoplasmic distribution of the fluorescence within the epithelial cells. General changes in rabbit-skin have been described in recent papers submitted by Linell (tar), Friedewald & Rous (tar and benzpyrene), and Berenblum (9.10-dimethyl-1.2-benzanthracene).

Untreated skin taken from the ear or from the back was stained for fat and the cornified layers assumed a reddish-yellow colour indicating a high fat-content, possibly emanating from the sebaceous

glands. No stained lipoids were however apparent in the actual epidermal cells. During the first 24 hours after the application of tar fat droplets or fat granules were observed in the cells within the stratum basale and the spinosum. They were accumulated around the nuclei, especially at the poles of the latter. The fat granules corresponded to the fluorescent granules and droplets visible in the fluorescence microscope. Repeated applications of tar were not followed by an increase in the number of the fluorescent granules and droplets, which as a rule disappeared 3 or 4 days after the tarring. No fat droplets were observed in skin treated with benzpyrene or 9.10-dimethyl-1.2-benzanthracene. In this respect the picture differed from that presented by the skin of the mouse but agreed with the pictures in the fluorescent microscope, where hardly any fluorescent granules could be seen.



Fig. 4.

Scattered fluorescent crescents and rings around the nuclei within the basal epidermal cells after two applications of 9.10-dimethyl — 1.2-benzanthracene.

In the present experiments, as was also the case in earlier studies on mice, fat staining could not explain the perinuclear accumulation of the fluorescence, whereas mitochondrial stains revealed valuable information in this respect. Normal skin excised from the ear or the backs of rabbits manifested only sparse mitochondria in the form of a few small granules scattered within some of the epithelial cells. After the application of the carcinogenic agents a definite increase in the number of mitochondria was observed, the increase varying according to the agent applied. The increase observed after the application of benzpyrene was but slight. During the 2nd and 3rd day after treatment some of the cells in the stratum basale and spinosum exhibited a sparse perinuclear accumulation of mitochondria, which were often manifest as a grey condensation of the cytoplasm around the nuclei. This condensation tallied fairly well with the faint perinuclear bluish-violet rings seen in the fluorescent microscope in some of the cells. The

repeated application of benzpyrene led to a numerical increase of the mitochondria, which was, however, never so conspicuous as in mouse-skin treated with benzpyrene. Also in other respects was the reaction of rabbit-skin to benzpyrene rather negligible. The epidermis showed only a slight increase in thickness, the individual cells were only slightly larger than normal, and the nuclear changes expressed by a swelling of the nuclei and enlargement of the nucleoli were insignificant.

Unlike benzpyrene, tar produced a pronounced reaction in rabbit-skin: 48 hours after being tarred the epithelium was hyperplastic and slightly hyperkeratotic, and in the course of the subsequent days the hair follicles were transformed into cups lined with hyperplastic and hyperkeratotic epithelium, and in some cases the border between the

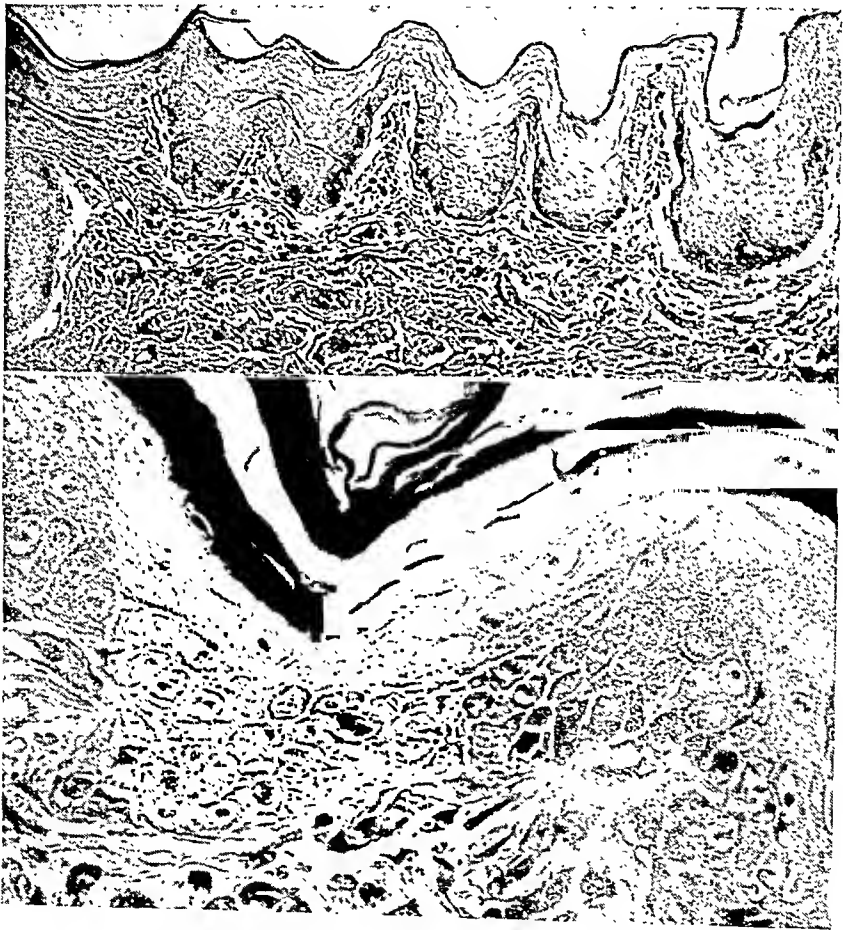


Fig. 5.

About 72 hrs. after the application of 9.10-dimethyl — 1.2-benzanthracene the basal cell layers show an increase in the number of mitochondria giving the cytoplasm a greyish-black colour. Mitochondrial staining ad modum Regaud.

epithelium and the corium was irregular. Repeated applications of tar caused a gradual increase of these changes. The tarring of the skin was soon followed by a manifest increase in the number of mitochondria, which was rather substantial after 48—72 hours. The mitochondria, resembling fine granules, surrounded the nuclei in the stratum basale and spinosum and gave the perinuclear zones of the cytoplasm in these layers a greyish colour in specimens stained *ad modum* Regaud. The localization of the mitochondria corresponded to that of the fluorescent rings but the former were not so numerous as the thickness of the rings might suggest. Tar contains several fluorescent substances which may possess affinity not only for the mitochondria but also for other cytoplasmic components in the perinuclear zone. Repeated applications of tar did not seem to be able to increase the number of mitochondria. On the contrary, they seem to decrease in number as soon as the hyperplasia of the epithelium has reached a certain stage.

The number of mitochondria in the epidermal cells, however, increased most after the application of 9,10-dimethyl-1,2-benzanthracene. The production of but a slight hyperplasia of the epidermis caused a definite increase in the number of mitochondria in the basal cell layers, which became more obvious as the epithelium increased in thickness. The cells within the basalis and the basal layers of the stratum spinosum then showed a dense perinuclear accumulation of mitochondria, and in some cells they filled the whole of the cytoplasm (Fig. 5). None of the carcinogenic hydrocarbons tested caused such a conspicuous numerical increase of the mitochondria in the skin of the rabbits as did this substance. The reaction was much more intense than in the tarred areas, in spite of the fact that hyperplasia of tarred skin may at times be more severe after the same interval subsequent to the application of the tar.

Discussion.

Of interest in the present investigation are the comparison between the fluorescence of benzpyrene in the skin of the mouse and that of the rabbit, and, secondly, the comparative effect of different carcinogens on rabbit-skin. As regards the first comparison the distribution of benzpyrene in the mouse differed obviously from that in the rabbit. In mice the benzpyrene accumulated in the perinuclear cytoplasmic zones of the epidermal cells and produced distinct fluorescent rings around the nuclei (Graffi; Hamperl, Graffi & Langer; Ahlström & Berg). In rabbits the benzpyrene soon entered the epidermal cells but was diffusely distributed in the cytoplasm or showed only a slight tendency to accumulate around the nuclei. This seems to suggest a correlation with the fact that benzpyrene has a rather strong carcinogenic activity in mouse-skin but a weak activity in rabbit-skin.

A comparison of the different carcinogens tested on rabbit-skin also seems to suggest a certain correlation between the carcinogenicity and the perinuclear accumulation of the agent. Tar, which is powerfully carcinogenic in rabbit-skin, produced a distinct perinuclear accumulation of the fluorescence. This was also the case with the strongly carcinogenic 9.10-dimethyl-1.2-benzanthracene. The analysis of the latter substance was, however, difficult on account of the weak fluorescence.

In the rabbit, as was also the case in the mouse, the accumulation of the fluorescent substance around the nuclei was correlated with the number of mitochondria in the epidermal cells within the treated areas of the skin. The numerical increase of the mitochondria was but slight after the application of benzpyrene, rather pronounced after treatment with tar, and very conspicuous after the skin had been painted with 9.10-dimethyl-1.2-benzanthracene. The increase in the number of mitochondria is not, however, a specific carcinogenic reaction. The reaction has been described even when the skin has been treated with different non-carcinogenic agents (*Berg*). The varying degree of the reaction after treatment with various carcinogenic agents only indicates that different hydrocarbons have a varying proliferative effect on the epithelium of the skin.

Judging by earlier experiments performed on mice and rabbits, it seems probable that the perinuclear fluorescent rings are due to an accumulation of the hydrocarbons or their fluorescent derivatives in the mitochondria. After the tarring the rings were more conspicuous and broader than might be expected from the number of mitochondria. This indicates that the perinuclear zones contain not only lipoids of the mitochondria but also other cellular constituents with affinity for the fluorescent components of the tar. The nature of these constituents is not known. It is, however, remarkable that 9.10-dimethyl-1.2-benzanthracene, which is the most powerful known carcinogen in rabbit-skin (*Berenblum*), also produced the greatest increase in the number of the mitochondria.

Summary.

Rabbit-skin, painted with an 0.5 per cent solution of benzpyrene in acetone showed a diffuse bluish-violet fluorescence in the cytoplasm of the epidermal cells. About 24—48 hours after the application of the benzpyrene the fluorescence was only slightly stronger in the perinuclear zone of the cells than in the outer zone. This phenomenon is unlike the reaction of mouse-skin in which benzpyrene or its fluorescent derivatives show a conspicuous perinuclear accumulation.

Skin treated 24—48 hours previously with tar showed a greyish blue fluorescence in the cytoplasm of the epidermal cells and sparse fluorescent granules in the stratum basale and spinosum. In addition

hereto the perinuclear zone of the cytoplasm showed a rather strong bluish-white fluorescence. The fluorescent granules corresponded to fat droplets in the cytoplasm. Compared with benzpyrene and tar, 9,10-dimethyl-1,2-benzanthracene produced only a weak, dark violet fluorescence in the epidermal cells. About 24 hours after the application of this chemical however, fluorescent rings could be distinguished around the nuclei.

The significance of the findings and their relation to the carcinogenicity of the substances tested on rabbit-skin is discussed.

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ANTIGEN — ANTIBODY REACTIONS IN GELS

By *Örjan Ouchterlony*.

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If an antigen and the corresponding precipitating or flocculating antibody diffuse towards each other in an indifferent gel of suitable consistency, e. g. gelatin or agar, there will appear, under certain circumstances a streak- or bandlike precipitation in the gel between the two diffusing components. The reaction is probably of the same nature as that between an antigen and an antibody. The position of this band of precipitation in relation to the two diffusion centres depends inter alia upon the initial concentration and the diffusion velocity of the two reacting substances. A similar reaction is obtained if antigens are allowed to diffuse in gels with a suitable constant proportion of antibodies or vice versa. In these cases the reaction appears at varying distances from the diffusion centre, depending partly on the concentration, the diffusion velocity and the time elapsed before the reaction becomes visible.

The fact that antigens and antibodies diffusing in gel react in this way was observed many years ago. In 1905 Bechhold (1) describes a reaction of this kind in 1 % gelatin between goat serum and anti-goat-serum obtained from rabbit. A similar observation is made by Nicolle, Cesari and Debains (2) in 1920 for diphtheria toxin and antitoxin from horse. Diphtheria immune serum as surface layer in a tube was allowed to react with toxin mixed with an equal part of 10 % gelatin. They then observed a disc-like precipitation of toxin-antitoxin character. In 1932—34 Petrie (3), Sia and Chung (4) and Kirkbride and Cohen (5) described specific and non-specific «halo» phenomena around bacteria colonies growing on substrates containing immune serum. Their investigations concern meningococci, pneumococci and Shiga dysentery bacilli. The method was later worked out for the serological typing of meningococci and pneumococci. In 1948 diphtheria toxin-antitoxin reactions of similar character were described by the author (6).

The object of this paper is to describe, by means of some model experiments, toxin-antitoxin reactions in solid and semisolid agar media.

Experimental.

Most experiments were carried out with diphtheria toxin and antitoxin. The former was culture filtrate of the strain PW 8, cultivated on Philippe Loiseau's medium. The latter was serum from a horse, immunized with toxin and toxoid, from such a culture filtrate. Growing bacteria of the same strain were used in some of the tests instead of the toxin already produced in the filtrate.

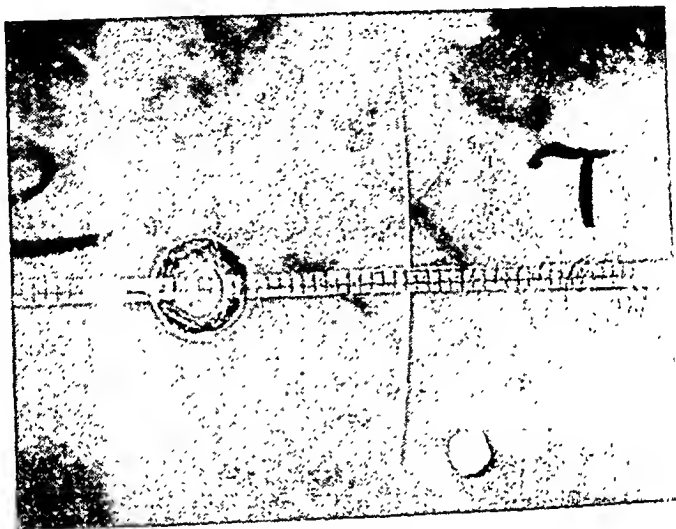


Fig. 1.

The experiments were made in Petri dishes (about 10 cm. in diameter) where both or one of the reacting substances were allowed to diffuse in an agar medium of varying composition. A thin layer of 2% washed agar was poured into each dish in order to obtain an even layer for the diffusion medium.

Test 1.

On a surface layer of serum agar (agar 1%, serum 50%) two so-called penicillin cups were placed at a distance of about 3 cm. from each other. One of the cups was filled with toxin (27 FIU/ml) and the other with antitoxin (45 FIU/ml). The plate was then incubated at 37° C. After 48 hours a thin line, which gradually spread laterally, could be seen between the two cups. Fig. 1 shows a reaction of this kind.

Test 2.

On a surface layer of serum agar to which antitoxin had been added (5 FIU/ml medium), a cup was placed and filled with toxin (25 FIU/ml). The plate was incubated at 37° C and after 48 hours a «halo»-like, sharply defined, bandlike precipitation was observed around the diffusion centre. The precipitation grew more and more distinct during the following days. Figs. 2 and 3 show the appearance of this kind of reaction.



Fig. 2.



Fig. 3.

Test 3.

The initial conditions were the same in this experiment as in test 2, but the cup was refilled at the same rate as toxin diffused into the medium. The time of observation was extended and under these conditions the halo was found to move slowly out from the diffusion centre. After two refillings the halo was 18 mm in diameter on the second day. After seven further refillings and nine days, the same halo was 20.5 mm in diameter.

Test 4.

The initial conditions were the same as in test 2, but there was a higher concentration of antitoxin in the plate. More or less sharply defined, multiple, concentric halo formations were observed by extending the observation time. The first, and most distinct halo corresponded to that described in the previous experiment. Multiple halo reactions of this kind are shown in fig. 4.

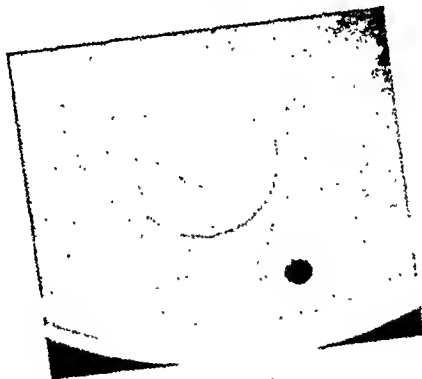


Fig. 4.

Test 5.

In the same way as in test 2, a series of immune serum plates were poured with media of decreasing antitoxin concentration 10 — 5 — 2.5 — 1.25 IU/ml. A cup with diphtheria toxin 25 IU/ml was placed on each plate. The plates were then incubated at 37° C. Halo formations appeared around the diffusion centre in the immune serum agar. The lower the content of immune serum the larger the diameter of the most distinct halo on each plate. The precipitation appeared first in the plate with the highest concentration of immune serum.

Test 6.

On an immune serum agar plate, prepared as in test 2, the strain PW 8 was spread. The plate was incubated at 37°. After 24 hours a »halo« formation appeared around the growing strain. This became more intense during the following days. Fig. 5 shows such a »halo« formation

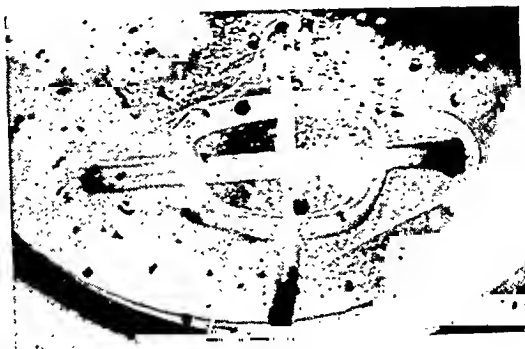


Fig. 5.

Test 7.

A diphtheria strain which had given varying results in toxicity tests *in vivo* was spread on a plate prepared as in test 2. The inoculum was taken from a culture which had given positive results in toxicity tests on guinea pigs. The plate was incubated at 37°. »Halo« phenomena could be observed after a few days around some of the growing colonies whereas others showed no such reactions even after continued observation. Positive results were obtained in toxicity tests on guinea pigs from colonies showing »halo« formations. Those without »halo« formations gave negative results.

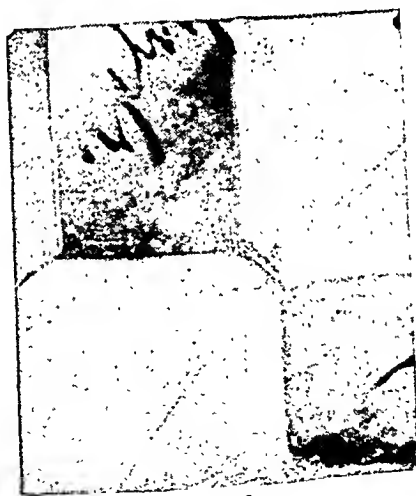


Fig. 6.

Test 8.

On a serum agar plate as in test 1 two basins (2 × 5 cm. in size), placed at right angles to each other, were cut out from the surface layer. One was filled with toxin 25 FIU/ml and the other with antitoxin 25 FIU/ml. The plate was then incubated at 37°. After 48 hours a streak was observed between the two basins. Several streaks appeared later. These streaks grew peripherally, later forming constant angles to the toxin and antitoxin basins. The most distinct streak formed an angle of 39° with the edge of the immune serum basin. Fig. 6 shows such an experiment.

Test 9.

A bottom layer of ordinary agar was poured into a Petri dish with high edges. When it had congealed a mixture of horse serum and ordinary meat infusion agar in equal parts was poured over it in a thin layer. After refrigeration, a two cm. strip was removed from the surface layer and replaced by serum agar to which diphtheria immune serum (200 FIU/ml substrate) had been added. The plate was then inoculated with the strain PW 8 in two parallel streaks at right angles to the »trench« and incubated at 37°. After 24 hours raylike precipitates were observed extending from the streaks of bacteria. Gradually these precipitates grew out peripherally and on meeting similar precipitates from adjacent strains, turned away from their original direction to confluate with them. Such precipitates interfering with each other are shown in fig. 7.

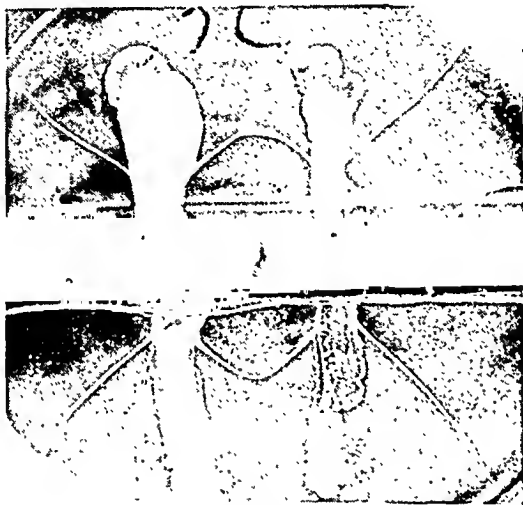


Fig. 7.

Test 10.

The same initial conditions were used as in the previous experiment. On this plate, PW 8, one atoxic and three toxic diphtheria strains, tested on guinea pigs, were inoculated side by side. After incubation for a couple of days, precipitates were extending from all of the five strains, those from PW 8 and from the toxic strains interfering with each other, whereas those proceeding from the atoxic strain crossed the toxin-antitoxin streaks of the toxinproducing strains without being affected. They did, however, interfere with a weaker streak, proceeding from PW 8. Interference and crossing phenomena of this kind are shown in fig. 8.

Discussion.

The reactions described above show a definite resemblance to flocculation reactions between toxins and antitoxins observed during experiments in tubes with liquid media. A certain time after mixing of the reacting substances the antigen-antibody compound precipitates, first in tubes, where the components react in optimal proportions. Around this flocculation optimum, a weaker and slower flocculation appears within a limited zone. By further addition of either of the reacting substances it is possible to dissolve floccules already formed.

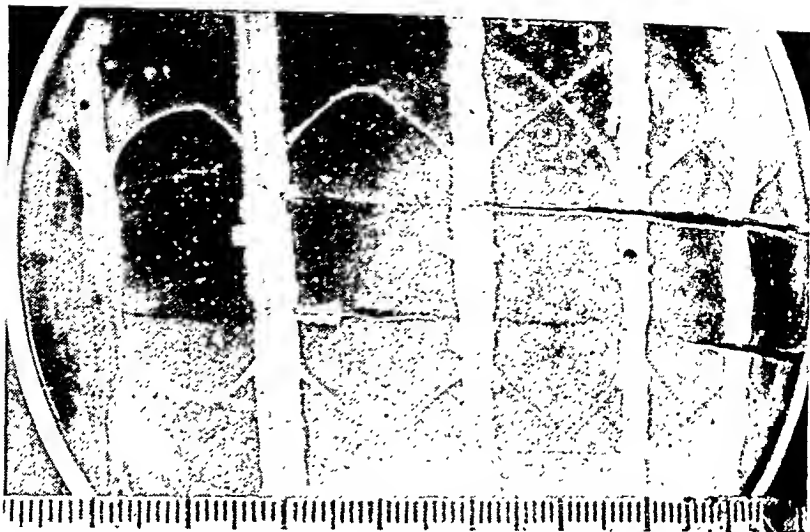


Fig. 8.

If, in tube tests, several antigen-antibody systems are simultaneously present, they react independently with regard to flocculation time, optimal proportions of the different systems etc. and do not interfere with one another. With diphtheria toxin and immune serum two different flocculation optima are sometimes observed. One of the flocculation reactions is a true toxin-antitoxin reaction and the other a so-called false flocculation, with a possible participation of a bacterial antigen and a corresponding antibody — A- and E-flocculation according to Schmidt and Scholz (7).

In diffusion tests in agar gels with diphtheria toxins and antitoxins, the distinct streaklike precipitation appears to be of toxin-antitoxin character, since it does not appear, if the toxin has been destroyed before the experiment, e. g. by heating. The position of the toxin-antitoxin streak in the diffusion medium is probably influenced by such factors as where optimal proportions between antigens and antibodies appear, the time necessary for the formation of a visible precipitate and the concentration — threshold value — of the reaction. In immune serum which contains several kinds of antibodies beside antitoxin multiple precipitates appear, somewhat less distinct however, than the toxin-antitoxin reaction. In the diphtheria immune sera examined, up to four antibody components beside antitoxin could be detected. By absorption of immune serum with appropriate antigens selective disappearance of the corresponding streaks of the precipitation spectrum was achieved.

The toxin-antitoxin precipitate in the agar seems to be soluble in excess of one of the reacting substances, as is the case with the floccules in liquid media. This phenomenon may possibly explain the re-

sults in test 3, where by a further addition of toxin, the radius of the »halo« increased considerably during the course of the experiment.

Tests 9 and 10 show that adjacent reactions between reacting substances of the same kind affect each other in such a way that the streaklike precipitations interfere with one another. That is not the case in antigen-antibody reactions of different kinds, where so-called crossphenomena appear instead. It is possible with the help of these interference- and crossreactions, to demonstrate serological difference or identity between different precipitating or flocculating substances.

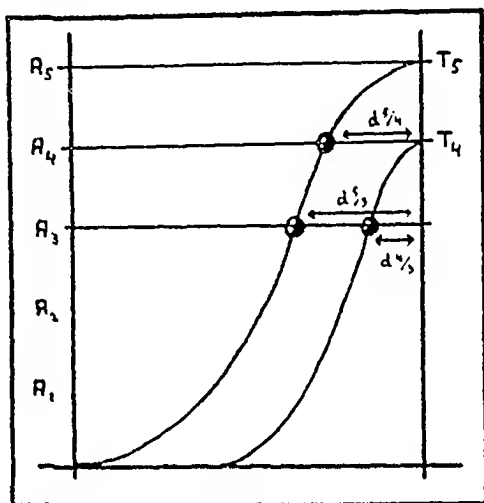


Fig. 9.

When antigens and antibodies diffuse at right angles to each other, it was furthermore noticed that there were only moderate variations in the angle formed by the precipitation streak with the directions of diffusion. For approximately equivalent quantities of toxin and antitoxin, as in test 8, the angle to the diffusion direction of the toxin was 38° — 39° . In multiple reactions it was observed that the angle for other precipitations differed in certain cases from that of the toxin-antitoxin reaction. Most often the origin of the precipitation streak also varied. The size of the angle and the situation of its vertex seemed to depend on such factors as the concentration, the diffusion velocity of the reacting substances and the proportions in which they combine.

As regards the sensitivity of the precipitation reaction in agar between diphtheria toxin and antitoxin it may be mentioned that visible, clearly defined streaks appeared in concentrations down to one FIU/ml.

The quantitative conditions of the reaction are demonstrated schematically in fig. 9. Toxin of a strength of T_4 and T_5 diffuse in media with a varying antitoxin content A_1 to A_5 . The reaction is pre-

sumed to appear at determined optimal concentrations of T and A. Optimal concentration in this method does not necessarily mean the same as optimal ratio in tube flocculation. The example given demonstrates the following.

Toxin with a lower strength than T_5 does not give any visible reaction in the medium with the content of antitoxin A_5 , lower than T_4 none in A_4 and so on, down to the sensitivity limit of the reaction which is about one FIU/ml.

With an antitoxin content of A_3 toxin strength T_5 gives a reaction farther away from the diffusion centre than with an antitoxin content of A_4 . Toxin of the same strength in media with successively lower content of antitoxin gives a precipitation more and more peripherally situated.

With an antitoxin content of A_3 , toxin of strength T_5 gives a more peripheral reaction than toxin of strength T_4 . With a constant content of antitoxin, the stronger the toxin tested, the more peripheral the reaction.

The examples illustrate that the concentration of the reacting substances are of decisive importance for whether and where the reaction will appear. Furthermore if the concentration of one of the substances is known, it is even possible, under certain conditions, to estimate the other substance quantitatively.

The application of the diffusion method thus also affords a possibility of making a quantitative comparison between the toxinproducing capacity of different diphtheria strains. The more strongly toxin-producing the tested strain is, in the higher concentration of immune serum does it give a positive reaction.

According to the facts stated above it is important to choose suitable concentrations of immune serum in the diffusion medium to obtain visible reactions. Since in practice there is often very little or nothing known about the toxinproducing capacity of the strains to be tested, it may be difficult to choose the adequate immune serum concentrations on using media with a constant content of antitoxin. A simple way of obtaining varying concentrations in the same medium within definite limits is, however, to allow the antibody to diffuse in a substrate, e.g. from a trench (tests 9 and 10) and to arrange the diffusion direction of the antigen at right angles to the antibody. This method does not, it is true, give the same possibility of estimating quantitatively the toxinproducing capacity of the growing bacteria, but it nevertheless offers other advantages. The simplicity of obtaining suitable immune serum concentrations has already been discussed. Furthermore, interference and crossreactions with known antigens contribute to more rapid and more conclusive results. The most convenient way to register the antigen components found by this method is to note their interference and crossing with known antigens and the position in relation to the toxin-antitoxin reaction as well as the angle to the diffusion direction of one of the components.

Tetanus bacilli, toxin and toxoid, gas gangrene bacilli and toxin, pneumococci and Dick toxin from streptococci have been tested by the same method and with similar results. The results of these investigations will be published later.

Summary.

Certain species of antigen-antibody reactions, e. g. between diphtheria toxin and antitoxin, may be made visible by allowing the substances to diffuse towards each other in a suitable gel, for example agar. Depending on such factors as combining proportions, concentration and diffusion velocity of the two components, streak- or band-like precipitates appear when the diffusion in the gel is suitably arranged. Combined with absorption methods, the diffusion method with interference and crossreactions allows qualitative antigen-antibody analyses. Quantitative classifications, within certain limits, are also feasible.

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IN VITRO METHOD FOR TESTING THE TOXIN-PRODUCING CAPACITY OF DIPHTHERIA BACTERIA

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The object of this investigation was to produce an inexpensive, simple, rapid and reliable in vitro method for the estimation of the toxinproducing capacity of diphtheria bacteria. The method in question is based on observations of toxin-antitoxin reactions in solid and semi-solid media (see the preceding paper). An account will be given in this paper of the in vitro technique and its results as compared with in vivo tests.

The material tested consisted of 2,039 strains of *Corynebacterium diphtheriae*. Most of them were freshly isolated from throat and nose swabs, sent to the Diphtheria Department of the State Bacteriological Laboratory for bacteriological examination. Clauberg-Herrmann's so-called indicator plates (dextrose-waterblue) containing 0.08 % potassium tellurite were used as a selective medium for the primary cultivation of the organisms. From this medium suspected colonies were cultivated on 10 % sheep blood agar. The diphtheria types were classified on the basis of the appearance of the colonies, haemolysis and their capacity of fermenting the carbohydrates, dextrose, sucrose and starch. In dubious cases other media as well, e. g. McLeod's, were used for the classification of types. The distribution according to types of the investigated material was 5.5 % *gravis*, 0.6 % *intermedius* and 93.9 % *mitis*. The toxinproducing capacity of all strains was tested by means of subcutaneous inoculation into guinea pigs. Pure cultures were seeded in tubes with 10 % serum broth and incubated for 48 hours at 37° C. in an oblique position. From such a culture 1 ml was then inoculated subcutaneously into guinea pigs of about 250 g. If the animals died a post-mortem was made and toxic lesions in the adrenals were registered. Surviving animals were observed for six to ten days after the inoculation.

The in vitro method, which was tested in several variations simult-

taneously with in vivo tests, is based on the observation that specific precipitates of toxin-antitoxin character appear when toxinproducing diphtheria bacteria grow on solid substrates containing a suitable quantity of immune serum. A more detailed account of this reaction has been given by the present author in the preceding paper. In the present investigation those strains were registered as toxinproducing, which gave rise to specific toxin-antitoxin or »halo« precipitations. Two immune sera were used, »Juno« with 450 FIU/ml and »Ljung« with 610 FIU/ml. Both were obtained from horses immunized in the usual way with diphtheria toxoid and toxin. No preservative was added to the sera, nor had they been heated.

Petri dishes of glass about 10 cm. in diameter and usually with high edges were used in all in vitro tests. A thin layer of 1.5 to 2 % meat infusion agar or water agar was poured into the dishes to compensate unevennesses of the bottoms. A satisfactory plain layer was thus obtained. Serum agar consisting of 1.5 to 1.75 % meat infusion agar mixed with 30 to 50 % cow or horse serum was poured into the dishes as a surface layer. Suitable quantities of diphtheria immune serum were then added to the surface layer in different ways. With regard to these variations of the technique the material has been divided into seven groups.

The following method, called the »S« method, was used for group 1 to 3. Varying quantities of immune serum were added to the surface layer before pouring, so that a series of plates with different concentration of antitoxin (40 to 0.63 FIU/ml medium) was obtained for each test. Four to six strains, as well as a strong toxinproducing strain PW 8 as a control, were then inoculated on each plate. The plates were incubated at 37° C. and read after 48 and 96 hours. After one and two weeks more in room temperature, the plates were again controlled. »Halo« phenomena of toxin-antitoxin character around the colonies were registered as a positive reaction.

The following method, called the »X« method, was used for groups 3 to 7. The surface layer of the plates was provided with a suitable content of antitoxin by letting the antibody diffuse from a trench containing serum agar to which immune serum had been added. The content of antitoxin in the trench varied for different tests (300, 90 and 40 FIU/ml medium). The strains to be tested were inoculated in parallel streaks at right angles to and across the trench. At least one streak of strain PW 8 was included in each plate as a control. The plates were incubated at 37° and read after 24, 48, 72 and 96 hours. By this method positive reactions appeared as thin raylike precipitates extending from the streaks of bacteria.

With the »S« method, the number of plates used, depending on the extent of the plate series (5 to 7 concentrations and a control plate without immune serum), was one to two plates for each strain tested. With the »X« method the number was considerably lower, since four

to eight strains as well as the control were inoculated on the same plate.

As to the modifications of the plate method used in the different test groups, the following observations were made.

In the first group 237 strains were tested by the »S« method. Seven antitoxin concentrations, 12.5 — 10.0 — 7.5 — 5.0 — 2.5 — 1.3 — 0.6 FIU/ml medium were used. In 92 % of the 126 toxic strains a positive reaction appeared within 48 hours, and in 8 % in 96 hours. No further positive reactions were observed on control of the plates after one and two weeks more. The highest antitoxin concentration of the medium in which specific »halo« reactions were observed was 12.5 FIU/ml in 31.7 %, 10.0 in 29.4 %, 7.5 in 21.4 %, 5.0 in 10.3 %, 2.5 in 6.3 % and 1.3 in 0.8 %. None of the strains had 0.63 FIU/ml as its highest reaction value. Calculated on the antitoxin concentrations 10 — 5 — 2.5 — 1.25 FIU/ml the majority of the strains showed a reaction in two or three consecutive serum concentrations. More exactly, in 14 % the zone width was one, in 65 % two and in 22 % three consecutive concentrations.

In order to find out the influence of potassium tellurite on the toxinproducing capacity of diphtheria bacteria, 308 strains — the second group — were tested simultaneously with the »S« method in two variations — media with and without potassium tellurite (S_T and S). Five antitoxin concentrations 40 — 20 — 10 — 5 — 2.5 FIU/ml were used. The potassium tellurite content used was 0.16 % or twice the quantity of the highest content in ordinary diphtheria selective media (Clauberg-Herrmann 0.08 %, McLeod 0.04 %, Clauberg II 0.035 %, Gundel and Tietz 0.033 %, Neill 0.02 % and Hoyle 0.01 %). The loss percentage as compared with in vivo tests (S — 2 % and S_T — 3 %) showed no significant difference between the two media (200 toxic

Table 1.

Immune serum concentration FIU/ml medium	S 40	S 20	S 10	S 5	S 2.5	Total	%
S_T 40		6	6	1		13	7.0
S_T 20		42	15	4		61	32.8
S_T 10	1	43	39	15		98	52.7
S_T 5	1	6	7			14	7.5
S_T 2.5							
Total	2	97	67	20		186	
%	1.1	52.1	36.0	10.8			

Group 2. Highest concentration of antitoxin giving positive reaction. 186 toxic strains.
 S_T and S = S technique with and without tellurite.

strains tested). The time for appearance of positive reactions was also approximately the same — 48 hours S in 83 %, S_T in 81 % and 96 hours S 17 %, S_T 19 %. After 96 hours no further positive reactions were observed during a week of continued control. As regards the highest antitoxin concentration in which positive reactions were observed (table 1), there existed a positive correlation between the two medias tested, with a slight displacement towards a higher concentration for the medium without tellurite. The zone width of the toxin reaction of the strains also corresponded in the two media (table 2).

Table 2.

Consecutive dilutions giving positive reaction	S 1	2	3	4	Total	%
S _T 1	14	22	11	1	48	25.3
2	24	42	33	2	101	53.1
3	6	20	10	1	37	19.5
4		4			4	2.7
Total	44	88	54	4	190	
%	23.2	46.3	28.4	2.1		

Group 2. Zone width of positive reactions.
190 toxic strains.

The majority showed reaction in two or more consecutive concentrations — S 77.9 %, S_T 74.7 %.

In the third test group, containing 283 strains, a comparison was made between the »S« and the »X« methods. On the »S« plates the same content of antitoxin was used as in group 2. In the »X« method a content of antitoxin 90 FIU/ml medium was used in the trench. As to the loss percentage — S 1.1 %, X₉₀ 2.6 % — no significant difference between the »S« and the »X« methods was observed for these 188 primary positive strains.

Since the »X« method was found to be very convenient for routine work, it was used alone in the fourth test group, consisting of 400 strains. The loss percentage for X₉₀ was 3.3 % of 164 primary positive strains.

In the fifth group, containing 285 strains, double tests were performed with the »X« method (X₉₀). The strains were tested primarily from the pure culture as in the previous groups, and again simultaneously with the guinea pig test and with the identical serum broth culture used for this in vivo test (X₉₀ and X₉₀G). On the primary tests of 120 toxic strains the loss percentage was 3.3 % with the »X« method. No difference between the results of X₉₀ and X₉₀G could be observed.

In order to investigate the presence and the frequency of precipitation reactions of non toxin-antitoxin character — so-called »false« toxicity reactions — the »X« method with two different antitoxin concentrations in the trenches — 300 and 40 FIU/ml substrate (X_{300} , X_{40}) — was used in the sixth group, consisting of 310 strains. For identification of the true toxin reactions, interference phenomenon, with a definite toxin reaction from strain PW 8, was used. This strain was inoculated at the same time and side by side to each strain which was to be tested. In 147 toxic strains tested the loss percentage was 13.6 % for X_{300} and 5.5 % for X_{40} . The use of a higher content of antitoxin in the trench caused a number of precipitation reactions, which were not of toxin-antitoxin character. X_{300} with 320 strains tested, showed such »false« reactions in 46 %. The corresponding value for X_{40} with 310 strains tested was 13 %. With X_{300} such reactions were observed in both toxic and atoxic strains. With X_{40} , however, they only occurred in atoxic strains (table 3).

Table 3.

	X_{40}			X_{300}		
	Toxic	Non-toxic	Total	Toxic	Non-Toxic	Total
Number of tested strains	141	169	310	127	193	320
Frequency of »false« reactions	—	39	39	65	81	146
%			13			46

Group 6. Frequency of »false« reactions.
310—320 tested strains.

In the seventh test group, containing 216 strains, the »X« method was employed with 40 FIU/ml. Of 134 primary positive strains the loss percentage was 9.0 % with X_{40} .

In 712 toxic strains tested (groups 3 to 7) positive reactions with the »X« method — X_{90} , X_{40} — appeared in 48 hours in 92.3 % and in 96 hours in 7.7 %. In the groups 5 to 7, where readings were made daily, the corresponding figures for 399 toxic strains were: 24 hours in 51.5 %, 48 hours in 39.3 %, 72 hours in 6.8 % and 96 hours in 2.4 %. With the »X« method it was thus possible to read a little more than nine tenths of the positive reactions after 48 hours and more than half of the reactions after 24 hours. This demonstrates quite a satisfactory agreement between the »S« and the »X« methods.

A summary of the results of comparative in vivo and in vitro tests for all seven test groups is shown in tables 4, 5 and 6. Of 2,039 strains tested about one half was found to be toxic and the other half atoxic.

Table 4.

Comparison between guinea pig test and plate method.
Results of primary tests.

Group	Method	Number of strains	G+ P+	G÷ P÷	G+ P÷	G÷ P+
1.	G					
	S	237	113	104	12	8
2.	G					
	S _T	308	183	106	6	13
3.	G					
	S, X ₉₀	283	181	95	5	2
4.	G					
	X ₉₀	400	157	236	2	5
5.	G					
	X ₉₀ , X ₉₀	285	112	165	4	4
6.	G					
	X ₃₀₀ , X ₁₀	310	133	165	4	8
7.	G					
	X ₄₀	216	121	82	12	1
Total		2.039	1.000	953	45	41
%			49.0	46.7	2.2	2.0

G = Guinea pig test

P = plate method

S = series of plates with successive dilution of immune serum

S_T = do. + tellurite

X = trench plate

Table 5.

Comparison between guinea pig test and plate method.
Loss percentage of primary tests.

Group	Number of toxic strains	G ÷		P ÷	
		Number	%	Number	%
1.	133	8	6.0	12	9.1
2.	202	13	6.4	6	2.9
3.	188	2	1.1	5	2.6
4.	164	5	3.0	2	1.2
5.	120	4	3.3	4	3.3
6.	145	8	5.5	4	2.8
7.	134	1	0.7	12	9.0
Total	1.086	41	3.8	45	4.1

G = guinea pig test

P = plate method

Table 6.

Comparison between guinea pig test and plate method.

Result of repeated tests of the strains non-corresponding in primary tests.

Group	Result of repeated test $G \div P \div$	Result of repeated test		Result of primary test $G \div P \div$	Result of repeated test	
		$G \div P \div$	$G \div P \div$		$G \div P \div$	$G \div P \div$
1.	12	7	5	8	8	—
2.	6	1	5	13	5	8
3.	5	3	2	2	2	—
4.	2	—	2	5	2	3
5.	4	—	4	4	—	4
6.	4	1	3	8	7	1
7.	12	9	3	1	—	1
Total	45	21	24	41	24	17
%		46.7	53.3		58.5	41.5

G = guinea pig test

P = plate method

A lack of conformity between the in vivo and in vitro methods was observed in the primary tests on 86 occasions i. e. in 4.2 % of the total material. Positive animal tests and negative plate tests were registered in 2.2 % and the reverse in 2.0 %. 1.086 strains showed positive toxicity reaction with both or one of the methods in primary testing. Of these strains 41 (3.8 %) gave negative animal test and 45 (4.1 %) negative plate test. In the different test groups the figures varied for negative animal tests between 0.7 % and 6.4 % and for negative plate tests between 1.2 % and 9.1 %. The loss percentage in primary tests thus shows no significant difference between the two methods (table 5).

Table 6 gives the supplementary examination of the 86 strains which showed divergent results on the first examination of in vivo and in vitro tests. This supplementary control was made from original cultures of the strains in question and with the same technique as in primary testing. The examination was repeated one to three times until uniform results were obtained with both methods. Of the 45 in the first animal test toxic strains 46.7 % gave positive results in following animal and plate tests. The remainder was found to be atoxic in both tests. Of the 41 primarily platepositive strains, 58.5 % gave positive results in both animal and plate tests when the examination was repeated. The remainder gave negative results with both methods.

The fact that only some of the primary positive results of the toxicity classifications were verified by supplementary examination can be explained in different ways. There exist, in in vivo as well as in in vitro methods, sources of error, which might cause misleading

results. More important in this connection, however, seems to be the variability of the diphtheria bacteria as to toxinproducing capacity. Primarily toxic strains with a great tendency to dissociate in atoxic variants often give negative toxicity results on repeated examination, since there are great possibilities that atoxic bacteria only are transferred in passages. In the preceding paper of the author, this opinion is also supported by the results of test number 7, in which the dissociation of a strain into toxic and atoxic variants was demonstrated by presence or absence of »halo« around single colonies when the strain was spread on a solid medium containing immune serum.

Finally, as regards tests on guinea pigs, a survey has been made concerning the survival time of the animals in definitely positive toxin reactions. A total of 1,045 strains pathogenic to guinea pigs were registered. 54.2 % caused death in 24 hours, 37.8 % in 48 hours, 6.1 %

Table 7.

	Guinea pig 2 days	Guinea pig 4 days	Total	%
Plate 2 days	594	63	657	92.2
Plate 4 days	47	8	55	7.8
Total %	641 90.0	71 10.0	712	

Group 3—7. Time of positive reaction.
Guinea pig and plate test.

Table 8.

	G 1 day	G 2 days	G 3 days	G 4 days	Total	%
X 1 day	87	77	21	5	190	51.5
X 2 days	63	71	8	3	145	39.3
X 3 days	12	8	5	—	25	6.8
X 4 days	7	1	—	1	9	2.4
Total %	169 45.8	157 42.6	34 9.2	9 2.4	369	

Group 5—7. Time of positive reaction.
Guinea pig and plate test /X technique/.

in 72 hours and 1.8 % in 96 hours. In 0.7 % the animals survived for more than 4 and up to 9 days.

Somewhat more than 90 % of the positive tests on guinea pigs showed results within 48 hours.

Considered the fact that in vivo tests include two days' cultivation in fluid medium, it is obvious that application of the in vitro technique in the diagnostic routine saves not only animals but time as well, the gain being approximately 48 hours.

Finally, the times required by individual strains to give positive results with the two methods were compared (tables 7 and 8). Of the 712 toxic strains from groups 3 to 7, — 594 showed early reactions (within 48 hours) with both methods. 8 gave consistently late reactions, whereas 110 (15.4 %) behaved irregularly. A similar agreement was found in a separate analysis of the results from the groups 5 to 7 with 369 toxic strains, where a more detailed study was possible, readings having been made with 24 hours' intervals.

Summary.

A toxin-antitoxin reaction in agar medium is elaborated as a routine toxicity test of diphtheria bacteria. In this plate method interference and cross reactions with known toxin producing strains make it possible to differentiate between true and »false« toxin reactions. The reliability of the in vitro method is approximately the same as that of in vivo tests on guinea pigs.

THE PATHOLOGY OF LAURENCE-MOON-BIEDL SYNDROME

By *Sven-Olof Brattgård*.

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The Laurence-Moon-Biedl^{1, 2}) syndrome comprises the following five symptoms:

1. Obesity,
2. Hypogenitalism,
3. Mental deficiency,
4. Eye changes of retinitis pigmentosa type, and
5. Syndactyly or polydactyly.

Of the some 300 cases of this disease hitherto reported in the literature only seven are autopsies (*v. Bogaert & Borremans*³), *Loepp*⁴), *Griffiths*⁵), *Riggs*^{6, 7}), *Radner*⁸), and *Anderson*⁹). The first case reported in 1936 by *Bogaert & Borremans*, included neither retinitis pigmentosa nor syn- or polydactyly. Of the post mortem findings, only those of the cranial cavity are mentioned. Polydactyly was also lacking in *Loepp*'s case, and the only reported findings are those in the sella tureica. *Riggs* gives a very incomplete clinical description of her two cases, and also her account of the autopsy findings is incomplete. In 1938 *Griffiths* published an autopsy case with a complete Laurence-Moon-Biedl (LMB) syndrome. The brain was carefully examined as was also the pituitary gland, but no cellular count was made. Here, however, as in other publications hitherto, no report is given of the histopathological changes of the eyes. In his communication *Radner* mentions the post mortem examination of a sister of the patient described in the following. Unfortunately the cranium was not opened. The most complete of all the autopsies seems to be the one performed by *Norman La Rue Anderson* (1941). The case was a full LMB-syndrome, and the pituitary gland as well as the brain was thoroughly examined, but unfortunately not the eyes.

As the etiology and the pathology of the LMB-syndrome have not

yet been properly explained the publication of thorough autopsies is highly warranted. The anatomical changes of greatest interest are above all those occurring in the brain, especially in the hypothalamus, the pituitary gland, and in the eyes. The skeletal and internal malformations often attending this disease, and the correlation of such malformations with the more central pathological changes also merit special attention.

The case described in the following is the younger sister of the autopsy case published by *Radner* (1940), who describes the hereditary and clinical pictures of the two sisters.

The sisters were born in 1907 and 1911 respectively. Earlier generations showed no evidence of anything pathological. The sisters were the third and fifth of six children. They had had an older sister with polydactyly and atresia ani, but she had died immediately after birth. The second and fourth children, who were boys, were apparently quite healthy. The sixth child, also a boy and likewise with polydactyly, died in infancy.

The older of the two sisters examined (G. N.) had adiposity, most pronounced around the trunk. Her sexual cycle was at first normal, but towards the end of her life she had had a constant uterine hemorrhage. Mentally, she had always been somewhat backward. At the age of fifteen her vision began to fail, and retinitis pigmentosa was diagnosed. She had small hands, stumpy fingers, and a supernumerary toe of postaxial type on either foot. In the spring of 1937 pyelitis developed, and a few months later she died with clinical symptoms of uremia. Autopsy revealed a moderate enlargement of the left heart, and an excessive shrinkage and cicatrization of the kidneys, the demarcation between the cortex and medulla being obliterated. The kidneys presented a picture of chronic glomerulonephritis. Double uterus and vagina. The cranium was not opened. Autopsy disclosed no further findings of interest.

The younger of the two sisters (A. N.), whose autopsy will be dealt with further down, was very adipose, especially around the trunk. This fat had initially begun to accumulate when she was seven years of age. She had her menarche at 20 yrs. In the beginning her sexual cycle was normal (3-4 day/4-6 week type) but later the interval became longer. During the last year of her life she had had a constant trickling uterine hemorrhage. The uterus was repeatedly curetted, and histopathological examinations gave the diagnosis »slight degree of cystic glandular hyperplasia«. As the uterine hemorrhage did not subside, roentgen-castration was performed in Nov. 1947, but the hemorrhage still persisted. Her standard of intelligence tested ad modum Wåhlén was found to be that of a nine-year old child. Psychically she was alternately hot-tempered and violent, and insinuatingly obliging. An examination of her eyes showed (Med. Dr. Stig Holm): Eye movement free and co-ordinated. No pathological nystagmus. Pupils equal in size, of normal size, regular, react well to light. Ophth.: yellowish, indefinitely delimited papillas with thread-like vessels. In the periphery numerous pigment foci, predominately resembling the »bone corpuscles« seen in retinitis pigmentosa. Fundus oculi pale greyish marbled, quite certainly due to chorioidal atrophy. The medias clear. Strabismus divergens oculi sinistri, 5-10 gs. Appear to be concomitant. The patient exhibits a peculiar tendency to close the left eye. Ptosis is not present. Vision, right eye = finger counting $1\frac{1}{2}$ m. Vision, left eye = finger counting 1 m. Field of vision could not be determined. Skiascopy right eye = 1.5 dptr. hyperopia. Skiascopy left eye = 1.0 dptr. hyperopia. Tonus = 18 mm. Hg. both eyes. The skeleton was mal-

formed inasmuch as the hands, especially the phalanges, were abnormally short. Radiograms of the hands showed no evidence of supernumerary skeletal parts. Each foot had six toes, the supernumerary being of postaxial type. The feet were conspicuously small. The radiograms revealed short metatarsal phalanges, especially the sixth phalang. The fifth metatarsal was stumpy and had two caput-like formations, against which the fifth metatarsal and the supernumerary toe articulated. The cerebral cranium showed nothing pathological. Sella turcica was overbridged, it measured 11×8 mm. on the roentgen film and was thus of normal size. In 1939 traces of albumen were detected in the urine and the sediment revealed a moderate quantity of white and red blood cells and granular cylinders. This urinary finding had been practically constant during the last eight years of the patient's life. Since 1939 the blood pressure had been about 200/140. On 5th. Dec. 1947 she died with typical symptoms of a chronic renal insufficiency with uremia.

Autopsy was performed 16 hours after exitus and revealed as follows:

Macroscopic examination:

The corpse of a somewhat short, thickset woman in her early middle age. A rich deposit of fat about the face, which was, however, not of »full-moon« or »mongol«-type. The extremities are somewhat short, especially the distal parts. No malformation of the configuration of the fingers. Each foot has 6 stumpy toes. Only five metatarsals are palpable. The arms and the legs appear to be rather thin because of the rich accumulation of fat on the other parts of the body. The subcutaneous fat of the abdomen is 5 cm. thick. Below the umbilicus, on lateral parts of the abdominal wall, and on the ventral side of the thigh, numerous white striae, a few centimetres in length, are to be seen. The skin is of a brownish-gray colour darkening distally until it becomes dark brown at the extremities.

The breasts are well-developed and adipose. The hair growth is normal and of feminine type. Labiae majora et minora as well as the vulva show nothing pathological.

Pericardium: normal. Heart: somewhat enlarged, wt. 370 gm with a slightly thickened left ventricle wall. Obvious, though slight, deposit of pericardial fat. The parenchyma is brownish-red and exhibits no gross changes. The cardiac openings are of normal diameter, and the valves as well as the cusps are also apparently normal. Coronary vessels are thin-walled and of normal calibre. Some atheromatous plaques are to be seen in the proximal part of the aorta, which was otherwise of normal appearance.

In the right pleural sac 200 cc. of clear, light, yellow fluid was found, and in the left, a few table-spoonfuls. The visceral pleura is smooth and glistening and free of adhesions. The lungs are of normal consistency except in the dorso-caudal parts, which are somewhat firmer. The cut surface of the lungs presents a normal picture except in the above mentioned parts, where compression of the tissues produces a moderate discharge of frothy fluid. Trachea and bronchi: normal.

Abdominal cavity: small quantity of clear, yellow fluid. The peritoneum is smooth and shiny. Esophagus, stomach and intestines: normal.

Liver: somewhat large, wt. 1600 gm. Smooth and even surface and of normal brownish-yellow colour. The cut surface shows a well preserved architecture which in some parts, however, seems to stand out too distinctly. Intrahepatic ducts: normal. Gall bladder: small with thick wall. It contains a light-gray, gelatinous substance. In the neck is a well anchored cholesterol stone. Biliary ducts otherwise normal.

Pancreas and spleen: normal.

Kidneys: embedded in somewhat large fat capsules. Both kidneys weigh altogether 110 gms. The fibrous capsule is adherent. The outer surface of the kidneys is covered with fine granules and is of a whitish-yellowish-red colour.

Some larger scars are also seen. The cut surface shows an outspoken reduction of the cortex, which is almost absent in some areas. The medullary region is relatively better preserved. Renal pelvis of normal size. Ureter and urinary bladder: normal.

Pituitary is normal in size and gross appearance.

The thyroid gland is enlarged, wt. 100 gm. Lateral lobes are nodular and are rather rich in colloid. The isthmus shows similar small nodules.

The adrenals weight altogether 22 gm. They show a fully normal picture.

The ovaries are rather large and firm and distinctly lobulated. They are of firm and tough consistence. Cut surface is homogeneous and whitish in appearance.

The fallopian tubes are normal in appearance.

The uterus is of normal size for the patient's age, and is without malformations. The cranial part of the uterine cavity shows evidence of a hemorrhage. No polypi or other gross changes are manifest in the uterus. Cervix uteri and portio vaginalis: nothing pathological. The vagina is of normal shape with pale, smooth mucosa. No malformations.

The cranium shows nothing pathological. The cut surface of the skull cap is normal. The spongiosa of the vertebrae is somewhat pale but shows no pathological signs. The compacta of the femur is of normal thickness. The middle of the femur contains fat marrow with a few, small reddish specks.

Brain: abnormally small, wt. 1070 gm. The meninges as well as the surface of the brain are apparently normal. The brain was fixed immediately in a 10 per cent solution of formalin.

Eye: removed and fixed in a 10 per cent solution of formalin.

Microscopic examination:

Liver: normal appearance. No signs of stasis, no necroses.

Kidneys: almost complete loss of the renal architecture. Only few of the glomeruli were preserved and even some of these show a thickening of Bowman's capsule. Most of the glomeruli show evidence of hyaline or fibrotic degeneration, especially the outer zones of the cortex, where practically all the glomeruli have been substituted by hyaline connective tissue, in which only scattered single nuclei are detectable. Here and there hypertrophic glomeruli with fused capillary loops are manifest. The convolute tubules have almost disappeared and are replaced by fibrotic connective tissue. Some tubules are dilated and show a preserved epithelium. Also in the marrow is there an increase in the interstitial connective tissue. Scattered collections of lymphocytes. The renal vessels show evidence of fibrotic degeneration and hyalinosis of the walls. Their lumen is reduced, sometimes obliterated.

The spleen and the pancreas exhibit nothing pathological.

The enlarged thyroid gland present the microscopic picture of a nodular colloid goiter.

Adrenals: normal proportion of medulla and cortex. The normally arranged cortical cells are surrounded by large capillaries and have a normal appearance. Ponceau-fuchsin stain shows no fuchsinophile granules.

Ovaries: marked fibrotic degeneration. Several atretic follicles are seen. One ovary has a large cyst containing a slightly stainable colloid, and lined with low-cylinder epithelium.

Uterine musculature is apparently normal. The mucosa shows nothing pathological.

The eyes: the sclera consists of a normal fibrotic connective tissue. The choroid seems to be somewhat thickened compared with normal material fixed in the same manner. In both of its vascular layers fibroid degeneration is observable, most conspicuously in the capillaries of the lamina vasculosa. Lamina chorio-capillaris shows nothing definitely pathological. Lamina basilaris is intact without any increase in size or signs of calcification. The

retinal pigmentary layer shows nothing unusual. The cone and rod cells are strongly reduced in number. The retinal changes (Fig. 1) are dominated by an exceptionally marked diminution of the outer nuclear layer. In some areas this layer has completely disappeared, other areas show a few surviving cells in immediate contact with the lamina basilaris of the choroid (Fig. 2). The inner nuclear layer is far better preserved than the outer but compared with normal material it shows a considerable numerical reduction. The layer of ganglia cells contains but a few cells and shows collections of pigment as the layer of the nuclear cells. These accumulations of pigment seem at least partly to be deposited around the retinal vessels. The latter also show evidence of arteriocapillary fibrosis.

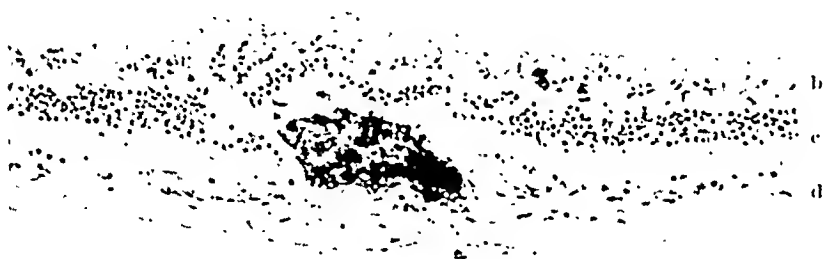


Fig. 1.

Pathological accumulation of pigment in the retina. (b = the reduced outer nuclear layer; c = the inner nuclear layer; d = the layer of ganglia cells).

The pituitary is sagittally divided on either side of the stalk. The resulting three sections are then examined at various depths. The relationship between the anterior and the posterior lobes is normal and the posterior lobe shows the usual picture with a few basophilic cells near the border of the anterior lobe. In the pars intermedia no colloid is to be found. A serial count ad modum *Mellgren*¹⁰) after staining with hematoxylin-eosin compared with a Mallory-staining gave the following percentage of the different types of cells: (number of cells counted: 16,565) Basophilic 17.7, Acidophilic 29.2, Chromophilic 53.1. Of all the basophilic cells 16.2 per cent are more or less vacuolized. No cells of the type described by *Crooke*¹¹) are observable. The relative distribution of the cells agrees well with that given by *Floderus*¹²), the eosinophiles being preponderantly central, the basophiles ventrobasal. In pars tuberalis and near pars intermedia a rich accumulation of small, rather light chromophobic cells are seen arranged in formations reminiscent of glandular tubules. This cellular type tallies completely with what *Krauss*¹³) termed 'embryonic cells'.

The brain, fixed in formalin, has narrow gyri and deep well developed sulci. It is sectioned ad modum *Schreidegger*¹⁴). Sections from the frontal and temporal lobes, as well as from the gyrus prae- and postcentralis exhibit normal structure and, compared with normal material, no cellular reduction. The area around the calcarine sulcus presents the picture of a completely normal structure with a pronounced line of *Gennari*. Number and appearance of the cells are normal. An examination of nucleus caudatus, nucleus supraopticus and the nuclei around the recessus opticus shows no differences when compared with normal material. The nuclei in the corpora mamillaria consist of nerve cells of a size peculiar to these nuclei. The large nerve cells in this nucleus seem to be numerically reduced compared with normal material. Nucleus subthalamicus consists of rather large nerve cells with a

cytoplasm containing a moderate amount of Nissl bodies in its peripheral region. The nuclei, which are localized somewhat excentrically in the cells, present a normal picture. The cells are perfectly normal in appearance but they seem to be considerably reduced in number. Sections of the medulla oblongata, the pons, and the cerebellum present normal pictures.

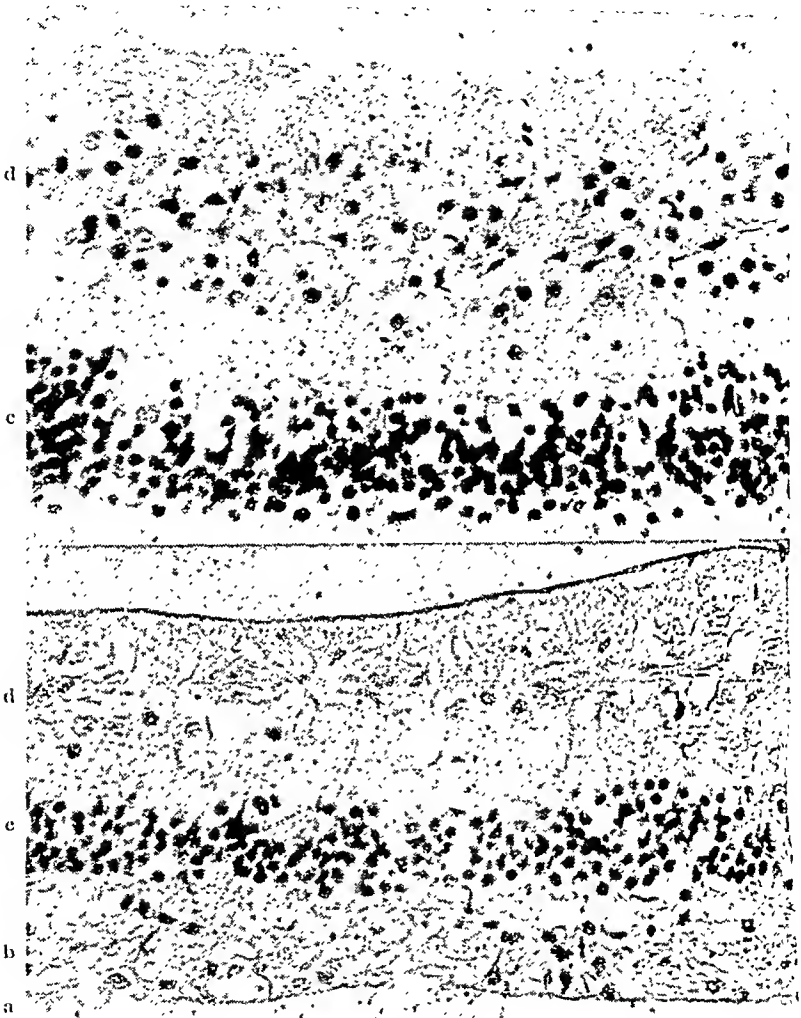


Fig. 2.

Retina shows a reduction of the layer of rods and cones (a) and of the outer nuclear layer (b). In the figure below the reduction of cells are much more pronounced also in the inner nuclear layer (c) and the layer of ganglia cells (d).

DISCUSSION

1. Pituitary changes in LMB-syndrome.

Owing to the similarity between the LMB-syndrome and dystrophia adiposogenitalis, interest in the pathophysiology of the syndrome has been concentrated upon the pituitary. This interest increased when *v. Bogaert & Borremans* (1936) found a hyalinosis of the pituitary

stalk in a case of LMB-syndrome. At about the same time *Loepp* observed a pituitary almost completely displaced by a cyst. *Griffiths* reported a pituitary of normal size and shape. He had, however, the impression that the basophiles had numerically increased at the expense of the eosinophiles. In order to get more exact information on this point *Anderson* (1941) counted the number of hypophyseal cells ad modum *Rasmussen*^{16, 17}) and found the number of basophiles to be 42 per cent. The normal number of basophiles according to *Rasmussen* is 11 per cent and the max. normal value 27 per cent. Numerous basophiles are found in hypertonia, in cystic kidneys and in uremia (*Berblinger*¹⁸), *Hawking*¹⁹), *Krauss*²⁰). All three conditions were present in *Anderson's* case. The pituitaries in the two cases reported by *Riggs* are said to have been normal, but no mention is made of a cellular count. In my case the cellular count was made ad modum *Mellgren*. This method can, as shown by *Mellgren*, be compared with that applied by *Rasmussen* and *Floderus*. In this case the basophilic component was 17 p. c., an admittedly high percentage (average in normals: 11.6 p. c.) but nevertheless within normal limits. This number is all the more remarkable because the patient died of uremia after having suffered for many years from renal insufficiency and hypertonia, and therefore might have been expected to show an increased number of basophiles. Whether the roentgen castration performed two weeks before exitus influenced the number of cells in the pituitary is highly improbable. The rtgn. dosage given for castration purposes generally has no effect on the uterine bleedings until 1—2 months after treatment, for which reason it is hardly feasible that the pituitary should manifest any substantial change already after 14 days. The so-called castration hypophysis with an abundance of small acidophilic cells found after complete surgical ovariectomy (*Rösle*²¹) cannot forthwith be used for the comparative study of hypophyseal changes occurring in association with rtgn. castration (*Joel*²²). In two cases of rtgn. castration of women a slight increase in the number of basophiles was found and a marked increase in that of the eosinophiles (*Berblinger*²³). Moreover, as the ratio between acidophiles and basophiles in my case was normal, there is every reason to assume that rtgn.-castration had not yet been able to bring about any essential hypophyseal changes. The so-called embryonal cells may, as pointed out by *Krauss*¹³), occur in normal pituitaries. Judging by *Riggs'* case and the one under discussion, it must be admitted that no correlation is discernible between the LMB-syndrome and hypophyseal changes — especially not an increased percentage of basophiles.

2. Cerebral changes in LMB-syndrome.

Gross anatomical changes are very scanty in LMB-syndrome. Except the shrinkage of the frontal poles mentioned in *Griffiths'* case, and the relatively small weight (1070 gm.) of the brain with thin gyri

and wide sulci in my own case, no other changes are reported in the literature. The microscopic changes are very difficult to distinguish and evaluate. No definite changes in the cerebral hemispheres in LMB-syndrome have hitherto been found. Of special interest is the occurrence of pathological changes in the hypothalamic nuclei, connected with the pituitary (*Harvis*²⁴). The positive findings made in these regions are a slight diminution in the number of cells in the supraoptic nuclei compared with the normal and a reduction in the number of glial cells throughout the hypothalamus (*Anderson*); fewer large cells than normal in the tuberal nuclei in the hypothalamus (*Griffiths*); and in my case, a numerical reduction of the cells in the subthalamic nucleus and a slightly decreased number of cells in the nuclei of the corp. mamillaria. *Anderson* also found slight changes in the molecular layer of the cerebellum. In none of the cases examined histologically could any traces of pathological changes be detected in the vicinity of the calcarine sulcus. *Riggs* thought that the cerebral vessels were markedly changed in his two cases, but no such degeneration is reported in any of the other brains examined.

3. Ocular changes in LMB-syndrome.

No histopathological examination of an LMB-eye has hitherto been reported. As the clinically observed eye-changes coincide exactly with those peculiar to retinitis pigmentosa, a comparison of the histological findings in these two diseases is motivated. Of those changes dominating the histological picture of the earliest cases of retinitis pigmentosa, the most striking one was a thickening of the choroid (*Wagenman*²⁵), (*Gonin*²⁶), (*Ascher*²⁷). This thickening was especially due to an increase in the thickness of the connective tissue of the choroid but also to a thickening of the vascular walls of the lamina vascularis (*Wagenman*, *Ascher*). The vascularization of this layer was also somewhat scanty. Many authors believed the number of capillaries in the lamina capillaris (Choriocapillaris) to be smaller than normal (*Stein*²⁸), (*Wagenman*, *Koyanagi*²⁹), (*Gonin*). The changes in the choroid were often most marked in those areas displaying the greatest retinal changes. In contradistinction to these researchers, who discerned pronounced changes in the choroid, (*Stock*³⁰) and (*Suganuma*³¹) described cases in which the choroid was practically normal. As to the retinal changes, the pathological picture is more consistent. The pigmentary layer was generally more or less normal but occasionally a slight proliferation of its cells (*Stock*) or a punctate or patchy complete destruction of the layer, could be observed (*Wagenman*, *Stock*, *Koyanagi*, *Ascher*). Frequently the layer is somewhat poor in pigment. All of the above cases showed a numerical reduction of the layer of the rods and cones and of the outer nuclear layer. They were preserved best in macular region, and worst in the periphery. In outspoken cases these layers seem to disappear (*Ascher*). Also the inner nuclear layer and the

ganglionic cell layer have been found to show a slight reduction. This reduction, however, is never so great as in the outer granular layer. The pathological accumulation of the pigment in the retina is described partly as extracellular (*Sugnumma*). The pigment is often collected around the retinal vessels (*Ascher*, *Suganuma*, *Koyanagi*). In many cases the vascular walls showed a fibrous thickening (*Stock*) with a narrowing, sometimes complete obliteration of the lumen (*Suganuma*).

The choroid of the LMB-eye in the present case was found to somewhat thick compared with that of normal eyes. The thickening was by no means so marked as in *Wagennan's* or *Ascher's* case. Neither were the vascular walls so abnormally thick as in their cases. Nor were the vascular changes distinguishable in the LMB-eye most conspicuous in those areas exhibiting the greatest retinal changes. The reduction of the layer of rods and cones and of the outer granular layer was, however, very severe (Fig. 2). Analogous with *Stock's* case the LMB-eye exhibited a thickening, though slight, of the walls of the retinal vessels.

4. Renal changes in LMB-syndrome.

In the incompletely described autopsy case of *v. Bogaert & Borremans* and *Loepp*, no mention is made of the appearance of the kidneys. In one of her cases *Riggs* pointed out the presence of glomerulonephritis but made no mention of the kidneys in the other case. *Griffiths'* case showed embryonal congenital malformation of the kidneys and in *Anderson's* case excessive renal cysts were observed. The older sister of the patient under discussion exhibited chronic glomerulonephritis, and my case presented the same picture. As renal malformations, even when slight, are liable to favour a disposition for renal inflammations (*Ask-Upmark*³²), *Runeberg*³³), *Strümpel*³⁴) it seems reasonable to assume the presence of an abnormal development or primary inferiority of the kidneys. As pointed out by *Ask-Upmark*, there seems to be a certain coincidence between renal and genital malformations, especially in women. Such genital malformations are very common in LMB, as will be apparent from *Panse's*³⁵) and *Cockayne's*³⁶) surveys. Also the case described by *Radner* and referred to above, had a vaginal septum.

5. Etiology.

The fundamental causes of LMB-syndrome are still unknown. That hereditary factors play a certain part is, however, beyond doubt (*Radner*⁸), *Cockayne*³⁷) etc.). The site of the anatomical lesion is, however, debatable. *Bardet*³⁸) assumed that the disease was due to a lesion of both lateral lobes of the pituitary. *Biedl*⁹) finding no roentgenological changes in the region of the sella turcica believed that the essential changes were to be found in the hypothalamic region or in the dience-

phalon. *Raab*³⁹) thought that the syndrome might be ascribable to a compression of the infundibulum disturbing the communication between the brain and the pituitary. In 1932 *Ornstein*⁴⁰) launched the hypothesis of the genetic origin of the disease. The disease is considered to be a genotypic inherited unit character defect. Adiposogenital dystrophy, renitis pigmentosa and mental deficiency are explained on the basis of a developmental defect of the ectopic zone of the prosencephalon for the embryological reason that the hypothalamus (infundibulum) and the optic chiasm take origin from the ventral segment and the end brain from the cephalic segment of the ectopic zone of Schulte. The other developmental anomalies appear because of the coupling of somatic genotypic defect characters with the cerebral units characters mentioned above. On the basis of his two cases *Riggs* believes the primary LMB-syndrome to be attributable to an early lesion in the cerebrovascular system, probably in the hypothalamic region.

Clinically, there is no difference between the eye changes of LMB and those peculiar to retinitis pigmentosa. As the histopathological pictures are also essentially similar, it might be of interest to dwell on the various theories of the etiology of retinitis pigmentosa. Many believe the cause of retinitis pigmentosa to be a vascular lesion. *Gonin* and *Wagenman* etc. believe the primary cause to be a lesion of the chorioidal vessels, which is, however, assailed by *Stock* and *Suganuma*. *Suganuma* assumes that the primary cause is to be sought in a lesion of the retinal vessels. That retinitis pigmentosa changes are never observed in emboli in the arteries of the eye (*Ascher*), in arteriosclerosis (*Koyanagi*), in renal lesions and secondary vascular disorders of the eyes (*Ginsberg*⁴¹), *Koyanagi*) argues against the theory of the above two authors. Some writers postulate the primary damage to be localized to the pigmentary layer (*Sugita*⁴²), an assumption that is, however, not supported by others. *Stock*, *Ginsberg* and *Collins* believe a degeneration of the neuroepithelial elements of the eye to be primarily responsible. *Stock* assumes either the presence of a cytotoxin, destructing the nervous tissues, or »einer kongenital zu schwachen Anlage«. (*Edinger's Aufbrauchtheorie*). *Collins* is inclined to believe in an abiotrophy, whilst *Ginsberg* believes a »mangelhafte Ausbildung« of the neuroepithelium to be primarily responsible. His cases had also testicular atrophy. *Schieck*^{43, 44}) is also an adherent of the primary damage of the epithelium of the retina being the cause. According to these last mentioned theories the vascular changes in the eye are secondary to the disorders in the neurogenic elements of the eye.

*Cunningham*⁴⁵) sought a physiopathologic explanation of the pigmentation of the retina in a disturbance in the hormonal regulation. Unlike the urine and blood of the controls those of patients with retinitis pigmentosa were able to produce a pigmentation in decerebrate frogs. He was able to produce a similar effect also *inter alia* in

pituitary abnormality. He believed this to be due to a melanosome-dispersing substance produced in a lesioned pars intermedia. *Zondek*¹⁶⁾ inclined rather towards the belief that the activity of this hormone facilitated the permeation of the pigment-carrying leucocytes emanating from the choroid and causing the pathologic accumulation of pigment in the retina (*Gasteiger*¹⁷⁾). In my case the examination of the eyes was unable to produce conclusive evidence as to which of the theories is the more probable. The generalized pigmentation of the skin gives more of interest, as it is more compatible with the *Cunningham's* theory than with *Zondek's*. Furthermore *Cunningham* has shown the melanosome-dispersing substance even in two cases with LMB-syndrome. As the pars intermedia is controlled by dienecephalonic centres it is obvious that a disorder in this part of the brain is also able to disturb the hormonal regulation in the pars intermedia.

The assumption of a primary vascular lesion being the cause of LMB-syndrome would render *Riggs'* theory compatible with *Wagenman's*, *Gonin's* and *Suganuma's*. The absence of cerebrovascular changes in *Griffiths'* case, in *Anderson's* as well as in my own and the relatively slight vascular changes I found in the LMB-eye argue, however, against *Riggs'* theory. The theory launched by *Bardet* and supported by *Griffiths'* and *Anderson's* examinations of the pituitary might possibly explain certain symptoms of the LMB-syndrome, but will not elucidate the eye changes. If the presence of a high number of basophiles in the pituitary really were the cause of the syndrome, one might have expected that many such cells would have been present also in my case. *Raab's* postulation that a disorder of the infundibular area is responsible for the syndrome is not better able to explain the change in the eye than is *Bardet's*, because not even gross pressure of the visual tracts is able to cause changes similar to those in pigmentosa. *Biedl's* assumption of a cerebral localisation of the damage is compatible with *Ornsteen's* theory. This theory may explain the hypophyseal symptom as a result of a disorder of the nerve centres controlling the adenohypophysis and located in the hypothalamic region, the mental deficiency may be explained by disturbance in »the cephalic area in the ectopic zone«. Can also the ocular changes be explained by disorders in this zone? According to *Stock*, *Ginsberg* and others, — and as far as the eyes are concerned, my case is similar to *Stock's* — retinitis pigmentosa is due to an anomalous development of the nervous elements of the eye. As these elements are derived phylogenetically from the zone in the prosencephalon mentioned by *Ornsteen*, it seems as if these theories are compatible for LMB and retinitis pigmentosa.

The material is too small to decide whether the renal lesions shown in the autopsy cases are incidental or whether they are primarily correlated to LMB.

SUMMARY

A description is given of the post mortem findings made in a case of Laurence-Moon-Biedl's syndrome in a 36 year-old woman who at the age of 7 years exhibited the first symptoms of the disease and who died with symptoms of uremia. Special attention is given to the occurrence of possible changes in the pituitary, the hypothalamus and in the eyes. The structure of the pituitary was histologically normal. Of the cells in the adenohypophysis 17 per cent were basophilic, 29 per cent acidophilic, and 57 per cent chromophobic. The number of basophiles lay within normal limits. The cells in the nucleus subthalamicus and nuclei corpora mamillariae were scanty, a finding tallying with that of earlier workers. The eye changes coincided with those met with in retinitis pigmentosa. Various theories of the etiology and the pathology of the syndrome are discussed. Attention is drawn to the fact that 5 of the 6 autopsy cases described exhibited renal changes.

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STUDIES ON BACTEROIDES

III. INVESTIGATION AND DISCUSSION OF METHODES

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In a previous publication (1) we have dealt with different aspects of the biology of *B. funduliformis* and especially stressed the fact that it is impossible with morphological and biochemical methods to distinguish between this microbe and another member of the *Bacteroides* genus, namely, *Necrobacterium necrophorum*.

In this paper we will discuss different strains belonging to the same genus, obtained from infections investigated by us, and we will criticize the different methods employed and try to establish them on a comparatively safe basis.

The material dealt with is as follows:

1. Specimen 1667. Pus obtained from a cervical abscess in a boy. The abscess had developed within a month without any general symptoms. Microscopically gram-negative elements as well as cocci in diploform were found. The former consisted of rods and threads of varying length. The rods showed segment-like structure after staining, had rounded ends and often round or elliptical structures as well as club forms. On cultivation the pus yielded hemolytic streptococci in aerobic cultures, while the anaerobic cultures gave a growth of hemolytic pleomorphic rods (*B. funduliformis* 1667).

2. Specimen 1596. Pus from a woman suffering from an abdominal abscess and suspected of tuberculous salpingitis. Microscopically numerous gram-negative elements were found of a strongly varying appearance: short rods, long rods and pleomorphic thread-like elements. On cultivation growth was obtained of *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and in anaerobic cultures *B. funduliformis* 1596, *B. fragilis* S. 14 and a weakly hemolytic strain of *Veillonella*.

3. Specimen 1721. Pus from an abdominal abscess in a young girl. The abscess had appeared in the right groin, in the patient who, 22 months earlier, had suffered from a diffuse peritonitis. Appendectomy had been performed

without any pathological condition being found in the appendix. On microscopical examination of the pus several gram-positive chain-forming cocci were found as well as gram-negative elements of pleomorphic appearance. Aerobic cultures gave growth of hemolytic streptococci, while the anaerobic cultures revealed *B. funduliformis* and a strain of *Veillonella*.

4. Specimen 10. Pus from a woman suffering from tympania uteri. In the pus gram-positive and gram-negative rods of varying appearance and length were found. Culture gave growth of *Lactobacilli*, *Corynebacteria* and *B. funduliformis*.

5. Specimen 958. Pus from a periurethral abscess in a woman. Microscopically numerous gram-negative rods. In anaerobic cultures growth of *B. funduliformis* in a pure culture (958).

6. Specimen 1251. Pus from an infiltrated area in the face. Microscopically numerous gram-negative rods and a few gram-positive cocci. Anaerobic cultures yielded a growth of *B. funduliformis* (1251), a gelatin liquefying microbe (not identified, strain S. 19), gram-positive cocci and a protein-splitting microbe (strain D. 10, not identified).

To avoid repetitions we will give a short survey of the criteria used in classification of our strains. Six strains of *B. funduliformis* have been isolated and compared to strains CH and 267 isolated by Prévot and to strain 748 isolated by S. Dick Henriksen. The characters justifying the claiming of our strains as *B. funduliformis* are as follows:

1. They grow only anaerobically with hemolysis of blood agar plates and form a metallic glistening halo.
2. They are non-motile and rapidly produce indole and hydrogen sulphide.
3. They produce the pleomorphic elementary bodies characteristic of *B. funduliformis* either in pathological material or in cultures.
4. They are serologically related to the strains CH and 267 of Prévot.
5. There is practically complete identity in characters between our strains and those of Prévot (*Spherophorus funduliformis* Prévot).

The most important of these characters is No. 5. According to Prévot, some of the other characters mentioned would cause an alienation from *B. funduliformis* and a classification into some other group of *Bacteroides*. (e. g., *B. floccosus*, *B. mortiferus*, *B. freundii*, etc.). When, however, we find that our strains in all main characters are identical with the strains isolated by Prévot under the designation *B. funduliformis* (*Spherophorus funduliformis*) we feel completely justified in our diagnosis.

The microscopical picture of the material examined as a rule was such that the possibility of *B. funduliformis* being present was quite distinct. In specimens Nos. 1667, 1596 and 1233 a marked pleomorphism of the gram-negative organisms made this possibility very pronounced, while the pleomorphism was less pronounced in Nos. 958

and 10, and in No. 1721 only a rare individual form appeared different from the ordinary gram-negative rod. However, it must not be forgotten, that organisms other than *B. funduliformis* may show polymorphism, and therefore it may hardly be correct to consider an organism as *B. funduliformis* on the basis of the morphological picture alone.

Strain S. 14 has been listed as belonging to the species *B. fragilis*, as all its characteristics coincide with those described by Prévot and by Bergey et al. (3). This strain consisted of small gram-negative rods with bipolar staining. It showed a slow growth with a homogeneous and delicate clouding of the broth and no difference of growth was seen in poor or in rich media. No production of indole and no gas in carbohydrate media. Acid was produced in lactose and strong coagulation was found in milk. The strain was motile.

Strains S 7, S 18, 1596 h++ and 1596 h+ were all reckoned as belonging to *Veillonella*, as they consisted of gram-negative cocci. 1596 h++ and h+ were hemolytic strains, while the others were non-hemolytic.

Strain S 19 was characterized by the liquefaction of gelatin, and strain D 10 by its lysis of protein.

When considering the importance of these microbes as etiological agents in the pathological conditions present, it is necessary to call attention to the fact that all but one of our samples contained other microbes beside the *Bacteroides*. No. 1596 contained *Staphylococcus aureus* and *Mycobacterium tuberculosis*, Nos. 1667, 1721 and 1233 showed hemolytic streptococci and No. 10 gave growth of gram-positive apathogenic rods. Only one specimen (No. 958) showed a pure growth of *B. funduliformis*. In order to obtain some evidence of the hypothetical significance of the strains of *B. funduliformis* to the disease, a complement-fixation test was performed with the different strains as antigen, and the sera of 958 and 10 as the antibody-containing sera. Serum 958 was held to be the most reliable one since this strain was found as the sole microbe in its specimen. The results of the tests were as follows:

Strains (Antigens)	Sera from patients	
	958	10
	Titers of reaction:	
1667	80	40
1596	80	20
1721	80	40
958	320	80
1251	80	80
10	160	80
CH (Prévot)	160	80
267 (—)	320	40
748 (Sv. D. H.)	160	40

In sera from patients 1596 and 1251 no fixation of complement was found. Thus, only two sera, i. e., from patients 958 and 10, showed a positive complement fixation, the former to a fair degree with its own strain and with the two certified strains from the Pasteur Institute and the strain previously identified in our own institute. Thus, it is probable that patient 958 has been infected with this strain and that it has caused the disease. It is more difficult to be certain as to patient 10, as this serum shows lower titers. However, the strain gives a fair titer in serum 958 and thus may be considered as identified serologically; the low titers (1:80) may indicate an infection due to strain 10.

None of the other sera gave any complement fixation with any of the isolated strains.

Most of the cases examined by us are derived from pathological conditions generally considered to be localities of choice for the anaerobic rods such as abdominal and genital lesions in women, lesions in the face and throat. It is well known that anaerobic rods are abundant in the intestines, in the female genital tracts and in the mouth. It is therefore quite reasonable to assume that, under favourable conditions, these organisms may gain entrance to the tissues of the organism and act as a real infective microbe. But it is also just as reasonable to assume that they may gain entrance through a mucous membrane that is injured by the infection set up by another and more active microbe and thus act as a secondary invader. It is also quite possible that the occurrence of these microbes in pathological material together with such microbes as streptococci and tubercle bacilli simply may be taken as an indication of an impurity of the material like that found in cultures from the nose consisting of *Staphylococcus albus* or some kind of *Corynebacterium*.

Cultural Investigations.

The gram-negative anaerobes isolated in this investigation have seldom been thoroughly examined as to their cultural behavior. We have therefore taken this opportunity to make a comparison between them. The growth is examined in peptone water, ordinary broth and double-strength broth. These media will give no growth of the organism in question under aerobic conditions. However, all the media are slightly reductive, and if the air is driven out through boiling they will reduce methylene blue for a short time. Addition of reducing chemicals to the media will increase and prolong this slight reducing power so that organisms not too intolerant of oxygen may grow in these media even in the open air. Such reducing compounds are glucose and hydrochloride of cysteine both of which are used by us. Böe thus found a promoting effect of cysteine towards *Fusobacterium*, when he used 0.01--0.03 % in 1 % glucose broth. Lohelle (3) found that

cysteine gave good growth of *Necrobacterium necrophorus* in concentrations of 0.001—0.05 % in double-strength broth. While Bøe assumed that *Fusobacterium* uses some of the cysteine in its metabolic processes, Lahelle held the effect of this compound towards *N. necrophorus* to be a purely reducing effect upon the medium. We have now tried to carry the results of Bøe and Lahelle obtained in our institute a step further in testing these compounds in the cultivation of several other *Bacteroides* and eventually demonstrating that media containing these compounds are suitable for the cultivation of all gram-negative anaerobic rods.

When a comparison is to be made between the effective concentrations of cysteine and glucose necessary for the growth of various microbes, it is most practical to use media in which the microbes cannot grow without these compounds. When the same basic medium is used, however, it may happen that microbes less sensitive to oxygen than others may grow so well that it will be difficult to see when the addition of the compounds has taken effect. We have therefore used as basic media such media in which the different strains cannot grow without the said compounds, and as the different strains are more or less sensitive to oxygen the basic media are chosen to suit this varying range of sensitivity.

Table 1.
Growth after addition of cysteine hydrochloride.

Strain	Medium	% cysteine-hydrochloride															
		.003	.004	.005	.006	.007	.008	.009	.010	.012	.05	.03	.07	.08	.10	.12	.14
S 19	1 % peptone water	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—
S 7	broth + 1 % glucose	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—
S 18		—	—	—	+	+	+	+	+	+	+	+	+	—	—	—	—
1596h+	broth	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
1596h++		—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
Frag S 14	broth	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—
267	1 % peptone water	—	—	—	+	+	+	+	+	+	+	+	—	—	—	—	—
CH		—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—
748		—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1596		—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—

Table I shows that cysteine has a promoting effect upon the growth of all our strains investigated. They have all grown in the concentrations of 0.01—0.06 per cent and as a rule the effective doses of cysteine are found to be 0.005—0.1 per cent. It is obvious that the less sensitive strains will react to smaller concentrations of the compound than the more sensitive strains — as we had expected. A distinct upper border-

line characteristic of the more or less sensitive strains has, however, not been found. It seems probable that *B. funduliformis* can use cysteine as its nutrient, while S 19 lacks this faculty. In spite of this,

The result of our experiments with cysteine is that all our strains. Consequently, it seems reasonable to assume that the function of cysteine in anaerobic culture chiefly is to produce a favourable reduction potential and counteract the effect of oxygen in the cultures, not to act as nourishment for the bacteria.

The result of our experiments with cysteine is that all our strains will grow when concentrations of cysteine between 0.01 and 0.06 per cent are present in the media. Bøe, Lahelle and the authors have found that increasing strengths of cysteine very soon show an inhibition of growth. It is therefore advisable to keep well within the border-lines of the effective concentrations of this compound.

The effect of glucose upon the growth of gram-negative anaerobes has also been investigated by Bøe and Lahelle. Bøe found a distinct effect upon *Fusobacterium* in concentrations between 0.1 and 10 per cent and an optimum between 0.75 and 4.0 per cent. Lahelle found *Necrobacterium* to grow in concentrations 0.1 and 7.0 per cent, with an optimum of 0.75—4.0 per cent. As will be seen in Table II, our own results corroborate those arrived at by Bøe and Lahelle. Our figures relating to the promoting effect of glucose lay between 0.6 and 3.0 per cent. All strains examined by us fermented glucose, but it did not seem as if the role of glucose as a nutrient to the microbe was the most important, since it would then have been difficult to explain why smaller amounts of glucose than 0.6 per cent had no effect upon the growth of the microbes.

It is a generally accepted fact that the inoculum in anaerobic cultures should be rather large. The reason seems to be that anaerobic bacteria produce reducing substances from the medium and, on inoculation into a new medium, part of these substances is transmitted with the bacteria and adheres to them as a protecting envelope, shielding them against deleterious influences from oxydizing substances in the medium. Hence the inoculum should be larger in poorly reducing media than in strongly reducing ones. This fact will necessarily be of interest when the growth factors required by anaerobic bacteria are investigated. It will not be correct to state the faculty of growth in a distinct medium for one generation only. It is clear that some of the products from the old culture carried over to the new culture with the inoculum may help the microbe in growing and thus mask the result of the test in question. On the other hand, the growth factors necessary to a certain microbe should be given after several generations on the same medium, using moderate inocula (Tables III and IV). It will be seen that all our strains show a greater reluctance to grow in several generations on a distinct medium than they do after a single inoculation from a brain-heart culture. None

Table III.
Growth in one generation.

Strain	Medium													
	1 % pep- tone water		broth				1 % glucose broth				cysteine glucose broth			
	boiled		not boiled		boiled		not boiled		boiled		not boiled		boiled	
	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline
S 19	+	—	+	+	+	+	+	+	+	+	+	+	+	+
S 7	—	—	±	—	+	—	+	+	+	+	+	+	+	+
S 18	—	—	±	±	+	±	+	+	+	+	+	+	+	+
1596h+	—	—	+	+	+	+	+	+	+	+	+	+	+	+
1596h++	—	—	+	+	+	+	+	+	+	+	+	+	+	+
Frag S 14	—	—	+	—	+	—	+	—	+	—	+	+	+	+
267	—	—	—	—	+	—	+	—	+	+	+	—	+	+
CH	—	—	—	—	+	—	±	—	+	±	+	—	+	+
748	—	—	—	—	—	—	—	—	+	+	+	—	+	+
1596	—	—	+	—	+	—	+	—	+	—	+	—	+	+

of our strains could grow in 1 per cent peptone water for several generations, while one strain managed to grow for one generation after inoculation from a brain-heart culture when the medium was boiled and covered with a vaseline seal. In the other media used (Table III and IV) the ability to start growth is distinctly different. This may be due to a different demand of nutrient in the different species. However, it seems more reasonable to explain the variation in behavior as a result of different sensitivity to oxydative processes occurring within the media, and that growth is more easily started as the reducing processes increase in the media. Thus, growth is always better in boiled than in raw media and better in media covered with a vaseline seal. When the media are thoroughly reduced, for instance, in boiled broth containing glucose and cysteine, all strains grow abundantly. These results show clearly that all the above-mentioned promoting compounds act through a reductive process and not as nutrients to the bacteria.

pH measurements in fermentation reactions in cultures of gram-negative anaerobes in broth as well as in sugar solutions.

The measurement of pH carried out with the aid of an electric potentiometer is more reliable in anaerobic work than the use of color indicators, especially since the latter method is unsuitable to point

Table IV.

Faculty of continuous growth in 10 generations on different media.

Strain	Medium													
	1 % pep- tone water		broth				1 % glucose broth				cysteine glucose broth			
	boiled		not boiled		boiled		not boiled		boiled		not boiled		boiled	
	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline	with vaseline	without vaseline
S 19	—	(2) —	+	—	(2) +	—	(5) +	+	+	+	+	+	+	+
S 7	—	—	—*	—	—**	—	—	—	+	+	+	+	+	+
S 18	—	—	—*	—	—**	—	—	—	+	+	+	+	+	+
1596h+	—	—	+	—	+	—	+	+	+	+	+	+	+	+
1596h++	—	—	+	—	+	—	+	+	+	+	+	+	+	+
Frag S 14	—	—	—	—	+	—	+	—	+	+	+	+	+	+
267	—	—	—	—	—	—	—	—	+	—	+	—	+	+
CH	—	—	—	—	—	—	—	—	+	—	+	—	+	+
748	—	—	—	—	—	—	—	—	+	—	+	—	+	+
1596	—	—	—	—	—	—	—	—	+	—	+	—	+	+

+: continuous growth for 10 generations. Inoculum 0.1 ml. medium.

—: continuous growth not possible with an inoculum of 0.25 ml. medium.

*: continuous growth for 10 generations after inoculation of 0.25 ml. medium.

**: continuous growth for 10 generations after inoculation of 0.15 ml. medium.

Figures in brackets indicate the number of the culture where growth did not occur after inoculation of 0.25 ml. of medium.

out such weak differences in the pH as may be met with in this work. Table V gives the results of our experiments along this line. All our strains have produced acid both in broth alone and in broth to which glucose and maltose had been added. In order to find the splitting of a certain kind of carbohydrate it is therefore necessary to examine a control culture in the basic medium without the carbohydrate in question. It will be seen from Table V that our strain S 19 when growing in pure ordinary broth reaches a pH of 6.4, which is almost identical with the value reached by *B. funduliformis* in glucose broth, namely, 6.38. *B. funduliformis* has always shown a weaker faculty to produce acid in carbohydrates than our other strains. All the other strains will reach values very close to pH 5, while *B. funduliformis* does not come below 6. If therefore this really means a splitting of glucose on the part of *B. funduliformis*, the carbohydrate cannot very well be completely hydrolyzed. It might perhaps be that the glucose

Table V.
pH in different media.

Strain	pH in media						
	Double-strength broth with addition of						
	Broth **	1% glucose	1% glucose **	1% glucose **	1% glucose + 1% cyst.	1% glucose + 1% cyst. **	1% glucose + 1% cyst. from **
S 19	6.10	5.45	5.07	5.10		5.40	5.08
S 7	6.85	6.00	5.45	5.22	5.45	5.35	5.50
S 18		5.60		5.46	5.40	5.27	5.22
159th *	7.10	6.04	5.37	5.32	5.27	5.22	5.17
129th *	6.90	5.30	5.20	4.63	5.01	5.01	5.02
Frage S 14		6.23	5.50	5.53	5.65	5.30	5.20
267	6.65	6.40	6.05	6.10	5.90	5.50	5.05
CH	6.80	6.04	5.50	5.87	5.52	5.42	5.20
748	6.75	6.27	6.00	6.05	6.15	5.70	5.45
1596	6.72	6.35	6.28	6.15	5.65	5.00	5.55

*. not covered with vaselene.

** covered with vaselene.

is used by *B. funduliformis* as a means of producing the right oxydation-reduction potential, thus inducing the microbe to a more vigorous growth with the production of acid from the broth alone. It is, however, not to be doubted that *B. funduliformis* is able to split glucose. This may be seen in fermentation experiments where cysteine is added to the glucose broth tubes. In these cultures *B. funduliformis* will reach a pH of 5. This cannot be explained as an acid production from cysteine, but must be caused by an activation of the glucose-splitting enzyme of *B. funduliformis*. Other investigators (Weil, Kocholaty and Smith 1939 (4) state that certain proteinases in certain *Clostridia* reached their maximal function only in the presence of cysteine.

Production of H₂S.

The presence of hydrogen sulphide in bacterial cultures is generally demonstrated by addition to the cultures of salts of heavy metals such as iron or lead. Addition of iron salt produces black sulphide of iron, whereas a greyish-black sediment of lead sulphide is formed in the cultures on addition of lead salt, when H₂S is present. The metal salts may be added to solid or semisolid media, such as lead acetate agar or the ferrichloride gelatin. H₂S may also be demonstrated in fluid media by the use of filter-paper moistened with a solution of lead acetate. This demonstration should consequently be simple and

clear — a black decoloration of the media should indicate the presence of H_2S , and the lack of such decoloration should indicate the absence of H_2S .

However, the demonstration of H_2S has always been an uncertain means of classification of bacteria and not to the least extent within the domain of the anaerobic bacteria. Bergey & al.'s Determinative Bacteriology contains many uncertain statements concerning the production of H_2S in bacteria and the same is also found in publications from the French investigation in anaerobes. It will be found that microbes may be »slight or non- H_2S producers«; some microbes are strong producers, according to some authors and non-producers according to others.

It is probable that the reason for such discrepancies must lie in the technique employed. It cannot be possible that a microbe can lose its power of production in passing from one institute to another. We must believe that the technique in such cases cannot be the *optimal* one. First, the microbe must be granted a supply of sulphurous organic material so that the production of sulphides will be distinct. It may be shown that the ordinary media for use in this connection very often contain only a very poor source of sulphur and they will often vary quite considerably in their content of sulphur. The consequence of this condition is that the demonstrable amount of sulphides may vary from one experiment to another and from one investigator to another, even if the ability of the microbe in question to produce H_2S is stable.

Another drawback with the ordinary media used for this demonstration is that they are often so poor that the growth of the microbe is minimal and consequently its biochemical functions stay at a low level. As all bacteriologists have often observed, clear-cut and distinct reactions with lead acetate or with iron chloride is under these circumstances very difficult or impossible to obtain.

As a rule, use of saturated filter-paper is unreliable. When many fluid cultures are grown in an anaerobic jar, and there are strong producers in this jar, the atmosphere within the jar will continuously be filled with H_2S and the gas will be absorbed by the filter-paper in culture tubes where no H_2S is produced, and the result of the experiment will be obscure.

To avoid all these pitfalls it is necessary that a medium suitable for the demonstration of H_2S must fulfill the following demands:

1. The medium must give an optimal growth.
2. The medium must contain sufficient sulphur to ensure a fair production of H_2S , if the microbe in question is able to do so.
3. The medium must be so composed that a distinct differentiation between positive and negative tests can be made.

To meet these demands we have used ordinary phosphate broth

with addition of 1.0 per cent glucose and 0.1 per cent cysteine in thick tubes containing 8 cc. of medium and provided with a piece of iron wire or an iron nail. The medium is boiled and cooled before inoculation. Under these circumstances the cultivation of the anaerobes may be carried out in the open air without the use of the anaerobic jar. If desired, the cultures may be covered with a layer of sterile vaseline for the demonstration of the development of gas. The growth starts very quickly in these cultures and the development of H_2S is demonstrated by the coal-black color that gradually takes the place of the formerly clear or slightly yellow culture fluid. In the case of a negative microbe the culture remains uncolored.

As to the favorable function of this medium it is obvious that both the glucose and the cysteine will bring about a favorable oxydation-reduction potential for the growth of the anaerobe. The iron will have the same effect and also function as an indicator of the presence of H_2S , which is fixed to the iron forming ferrisulphide. It is very probable that the iron favors the enzymatic process which results in the production of H_2S , but it is also possible that the fixation of the deleterious gas H_2S increases the possibility of growth within the culture and thus also increases the production of that same gas.

Table VI demonstrates clearly the superiority of our medium in tests for H_2S . Next to our medium comes the ferriehloride gelatin, while the lead acetate agar is very poor as a diagnostic medium.

Table VI.
Production of H_2S in different media.

Strain	B. H. + iron	D. S. broth + iron	Broth + gluc. + iron	Broth + gluc. + cyst + iron	Gelatin + iron	Gelatin + ironchloride	Agar + lead acet.
S 19	—	—	—	++ ⁴	—	+ ⁷	—
S 7	—	—	—	—	—	—	—
S 18	—	—	—	—	—	—	—
Frag S 14	—	—	—	++ ⁴	—	± ⁷	—
267	—	—	±	+++ ¹	++ ⁴	+++ ²	+++ ⁴
CH	±	±	—	+++ ¹	+++ ⁴	+++ ¹	+ ⁷
748	—	+	—	+++ ¹	++ ⁷	+++ ²	+ ³
1596	—	—	—	+++ ¹	+ ⁷	+++ ³	+ ⁷

The figures indicate the day of visible reaction.

The production of gas.

The ability of our strains to produce gas has been investigated in fluid cultures covered with a layer of sterile vaseline. Different media

have been tested, all media containing 10 ml., and the reading has been made after 7 days of growth.

The results are seen in Table VII.

Table VII.
Production of gas in different media.

Strain	Medium				
	Peptone water + 1% glucose	Broth	Broth + 1% gluc.	Broth + 1% gluc. + 1% cyst.	Meat
S 19	—	±	++	++	—
S 7	—	—	±	±	—
S 18	—	—	±	±	—
Frag S 14	—	±	++	++	—
267	++	++	++	++	+
CH	++	++	++	++	++
748	++	++	++	++	+
1596	++	++	++	++	+

Reactions in milk.

The reactions encountered in bacterial cultures in milk are of considerable importance in the correct diagnosis of anaerobic bacteria. However, the readings of these reactions are often quite difficult and the results of different authors not always comparable. We have therefore tried to correlate different reactions met with and have tried to adjust them to our material.

Milk is of course a very composite medium with its proteins, carbohydrates and minerals, and the different reactions met with from bacteria growing in this medium are caused by the different enzymes belonging to those bacteria. The types of reactions are as follows:

1. No macroscopic change of the medium.
2. Acid coagulation, caused by the flocculation of lactalbumen (isoelectric point 5.1) and of caseine (isoelectric point 4.6—4.9). If, therefore, the bacterial growth causes the pH to fall below 5, the conditions for an acid coagulation will be present. A starting coagulation will, however, as a rule take place when pH reaches 5.45—5.55.
3. Rennet coagulation (chymosin) caused by the enzyme not by the acid. As these two modes of coagulation are of different origin it is clear that the occurrence of coagulation in a certain test cannot be considered as a sign of the same biochemical procedure in all tests, but attempts ought to be made to differentiate between the two procedures capable of causing coagulation.
4. Peptonization of the milk as seen in a coagulum or, preferably, in a solid medium made from milk.

To meet these different considerations we have worked partly with fluid milk, partly with milk incorporated in agar. The former medium has been made from skimmed milk sterilized 3 times in the usual way in the Arnold apparatus. The inoculation has been made with 2 drops from a brain-heart culture of the microbe in question. If a culture made in this way and incubated in an anaerobic jar for 30 days showed no sign of morphologic change, it was ascertained whether there was any growth, and in the affirmative, the microbe was taken to have no effect whatever upon milk. If however, a daily control of the tubes in the experiment showed a starting coagulation the pH was immediately taken. If this was above 5.6, the coagulation was considered to be due to an enzymatic function (rennet or otherwise). If, however, the pH was lower when the coagulation started we held the coagulation to be due to acid production. However, it may happen that these two coagulations may occur in the same test. It is consequently necessary to make a special test for the occurrence of rennet in these experiments. This has been made in the following manner; The culture in question has been grown in double-strength broth for one week. Then the pH has been raised to 7.0 and the culture divided into three portions. One portion (A) was filtered through Berkefeld filters N, one portion (B) was centrifuged, the supernatant fluid discarded and the sediment taken up in saline to the original volume, while the third portion (C) was used unaltered in the experiment. To the clear filtrate A and the suspension B 1 drop of phenole to inhibit bacterial growth was added. To all three portions 1 ml. of skimmed milk was added. In this manner it was possible to decide whether there was any rennet production and if the enzyme was free in the fluid cell-free medium (A) or bound to the bacterial cells (B) whose respiration was hindered by the phenole only found in the living cells (C).

The peptonization of milk has been studied both in fluid and solid media of milk. The peptonization of caseine has been studied separately in a medium as employed by W. C. Frazier & Ph. Rupp (1928) (5) and made as follows:

- 3.5 gr. of caseine is moistened in 150 ml. water,
- 50.0 ml. Ca(OH)_2 ,
- 100.0 ml. 0.15 per cent Na citrate and
- 200.0 ml. ordinary broth are added.

The mixture is autoclaved, and an equal part of 3 per cent agar is added before use. This medium is opaque and an eventual peptonizing effect is demonstrated by clear zones round the colonies. As seen in Table VIII, this peptonizing effect upon the caseine does not seem to have any relation to the peptonization of the solid milk medium. The latter seems always to be related to an acid production of the strains as controlled in the fluid milk media (pH 4.5—4.9). The acid production in milk has been controlled by the production

Table VIII.
Reactions in milk media.

Strain	Media					
	Fluid milk			pH in peptone water + 1 % lactose	Agar + milk	Agar + caseine
	coag.	peptoniz.	pH			
S 19	+ ²	—	4.91	4.98	+ ²	—
S 7	—	—	6.26	7.05	—	—
S 18	—	—	6.48	7.14	—	—
Frag S 14	+ ¹⁰	—	4.61	4.96	+ ²	—
D 10	+	+	4.50	0	+	+
267	+ ³⁰	—	5.48	6.80	±	—
CH	—	—	6.32	6.95	—	—
748	—	—	6.48	6.83	—	—
1596	—	—	6.25	7.02	—	—

—: means no reaction after 30 days in fluid media.

—: means no reaction after 7 days in solid media.

+: means positive reaction.

of acide in 1 per cent lactose in peptone water and a complete correlation between the coagulation and acidification of lactose has been found.

Summary and Conclusions.

In this paper we have dealt with examinations of pus containing gram-negative rods and threads belonging to the genus of *Bacteroides*. In the course of these examinations we have tried to make some basic studies relating to the conditions of growth of these *Bacteroides* and also to ascertain the best manner of cultivation in order to obtain optimal growth and to distinguish between different species within the genus.

The *Bacteroides* cultivated by us occurred partly alone, partly together with aerobic bacteria of varying kinds. In order to ascertain whether or not the *Bacteroides* had any etiological relation to the diseases under examination, complement-fixation tests were performed. In two cases these gave positive results, indicating that our strains of *B. funduliformis* might be the etiological agent, while in the other cases negative results were obtained, thus indicating that the aerobic microbes might be of greater etiological interest, while the anaerobes must be considered as contaminants derived from the localities where abscesses had developed. This fact should always be born in mind when gram-negative anaerobes are encountered in purulent conditions with a mixed flora of microbes.

The biochemical examination of our strains gave some useful hints as to the cultivation of the above-mentioned kind. It has been shown that both glucose and cysteine hydrochloride are very favourable growth factors and able to initiate growth in poor media such as peptone water. A lower as well as an upper border-line of concentrations are very distinct. Cysteine functions at the concentrations of 0.006 — 0.1, glucose functions at 0.007—6.0 per cent on an average, while some strains require somewhat higher concentrations. When cultivated in ordinary broth a little less of these factors is necessary. The low concentrations necessary to initiate growth indicate that the factors may act more as promoters of a favourable reduction potential than as energy-giving factors.

Investigations as to the media suitable to give a continuous growth has been made in many generations of transfer, because a few generations are not able to show what factors are needed. Some growth factor may be carried over from the inoculations and give a wrong impression of the ability of the medium to sustain growth.

A point of great interest in fermentation reactions is very often overlooked in current literature dealing with these anaerobes, *i. e.* the pH measurement of the basic medium used with the carbohydrate in question and the same medium without this carbohydrate as a control. It may happen that this measurement may give the same figure in these two media, and thus it will be illusory to state that fermentation of the carbohydrate takes place under this condition. In the case of *B. funduliformis*, it has been demonstrated that the addition of cysteine may activate a latent glucose-splitting enzyme. A test without cysteine in this case would give a negative glucose-splitting ability, while a test with cysteine would give a positive result for one and the same strain.

The demonstration of H_2S in cultures of *Bacteriodes* plays a very great rôle in the diagnosis of species, as this demonstration in current literature very often is the determining factor placing a strain in one species or in another. It is a well-known fact to all bacteriologists that the ordinary technique on this point is very unsatisfactory. The question will often arise as to whether the test is positive or negative and a definite answer will not be possible. We have therefore worked out a new technique which always gives clear-cut results. This test is as follows: The strain in question is cultivated in a fluid medium (ordinary broth) with addition of cysteine and a small piece of iron. If H_2S is present a black decoloration is formed and H_2S may be chemically demonstrated, while the black sediment is seen to consist of sulphides. This test may be recommended to those working with these microbes.

The investigations of the milk-coagulating ability of strains is also of considerable interest. It may be seen from the literature that some authors claim that their strain either fermented lactose with a negative

coagulation test or vice versa. We have investigated this point very closely and have never found a positive coagulation test with a negative lactose fermentation. The reactions of our strains in solid milk media have also been investigated. Distinct clear zones round the colonies on such media always occur in strains giving coagulation of milk and acid production in media containing lactose. A correlation between reactions in solid milk media and media containing caseine was not found. The reactions of rennet-like enzymes in our strains did not seem to be of any value in the diagnosis of our strains.

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P. S. In No. II of this series (Vol. XXV, Fasc. 4, 1948, p. 691) we wrote that Weinberg, Nativelle and Prévot did not find indole in their cultures of *B. funduliformis*. This is a misunderstanding. They did find indole. We regret this incorrectness.

THE INFLUENCE OF THE ANTIGEN DENSITY AND OTHER FACTORS ON THE SERUM TITER IN THE AGGLUTINATION-LYSIS-TEST FOR LEPTOSPIROSIS

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Introduction.

A considerable disagreement between the results of the agglutination-lysis-test for leptospirosis performed simultaneously at two Swedish laboratories with one and the same dog serum, led us to consider the importance of different factors, primarily the antigen density, which might influence the determination of the serum titer by this test.

The relation between antigen density and serum titer in the agglutination-lysis-test for leptospirosis does not, so far, seem to have been studied systematically.

Most authors dealing with this test confine themselves to stress the importance of using young and well grown cultures for the test. However, what is considered a well grown culture in one laboratory may not be considered so in another laboratory. For instance *Broom* (1948) considers (formolized) cultures with about 10×10^6 leptospirae per ml suitable for the seroreaction, while *Savino & Rennella* (1943) work with (living) cultures containing $80-150 \times 10^6$ leptospirae per ml. It should be added that both *Broom* and *Savino & Rennella* perform their tests with mixtures of equal parts of culture and serum dilution.

Pettit & Erber (1936) use suspensions with about 100 (living) leptospirae per field (objective G, okular 4, Stiasnic) and dilute the cultures if they are «très riches». These workers perform the test with nine parts of culture and one part of serum dilution.

Gardner (1947) expressly advocates a standardization of the antigen density for his rapid microscopie test for leptospira agglutinins; according to *Gardner* the culture should contain at least 10 leptospirae per $\frac{1}{12}$ oil immersion field (the eyepiece magnification is not given), and if it contains more than 20 leptospirae per field it should be diluted correspondingly. The test is performed with about ten parts of culture and one part of serum dilution.

Stuart (1946) mentions a »standard density« meaning »a density considered suitable for serological work«, without giving further indications of the number of leptospirae in the »standard suspensions«.

Ward & Starbuck (1941) consider cultures with from 20 to uncountable leptospirae per field (magnification not given) »suitable as antigen for the agglutination reaction«.

For his macroscopic agglutination test Gardner (1943) prepared leptospira suspensions by the filtration through moistened cotton-wool of formolized three- to four-day-old serum-water cultures. Of these suspensions Gardner writes: »the density of suspensions does not vary greatly, and need not be standardized«, but no indication is given of the exact density of the antigen. By examination of Lederle's diagnostic »antigen« Gardner found that this antigen was six or eight times denser than his own antigen, and that the former seemed perfectly good for use in his method, when suitably diluted. The test was performed with equal parts of antigen and serum dilution (except in the first serum dilution (1 in 25), where practically undiluted antigen was used).

For the macroscopic agglutination-test described by Pot (1936) the antigen was prepared by re-suspension of the centrifugation-sediment from 250 ml leptospira culture in 10 ml formolized meat broth, and subsequent addition of formolized saline until the required density, »a definitely turbid reagent«, was obtained. The test was performed with mixtures of equal parts of antigen and serum dilution.

Smith & Tulloch (1937) for their macroscopic agglutination test used »young actively growing cultures«, and mixed six parts of culture with one part of serum dilution.

The above examples from the literature show that the density of the cultures used for the seroreaction in different laboratories (and sometimes perhaps also in one and the same laboratory) varies considerably. Consequently, if the antigen density influences the titer, the results of agglutination-lysis-reactions performed in different laboratories may not always be directly comparable, even when the same strains and the same technique, as regards serum dilutions, ratio of volumes of serum dilution and culture, incubation time and temperature, reading technique and end point determination*) are used.

It was, therefore, considered worth while to investigate further the relation between antigen density and serum titer.

Material and Methods.

Antigens. — 6 strains of leptospirae were used, viz. 2 *L. icterohaemorrhagiae* AB (M_{20} and M_{695}), 2 *L. sejroe* (M_{84} and M_{809}), and 2 *L. canicola* (Il. Utrecht IV and Alarieh). The first four were isolated from human beings in Denmark, the two canicola strains from dogs in Holland. Further details of the origin of these strains are given in table 1, part A.

Cultures were grown in Korthof's (1928) medium with c. 10 % filtered inactive normal rabbit serum, c. 0.1 % filtered hemolysed rabbit red blood cells, and c. 0.5 % autoclaved extract of baker's yeast (extraction 15 minutes in boiling water). 2 batches of medium, made with different batches of pooled rabbit serum, were used. The medium was inoculated with about 1/10 volume of one week-old cultures and incubated at 30° C for 5–21 days.

Dilutions of cultures were made with the same medium as that used for cultivation of the culture in question.

*) That divergences in serum titer must exist between laboratories, which use different end point determinations, is obvious.

Determination of antigen density. - Immediately before the cultures were used for titration experiments a small portion of each culture was formalized by adding 10 % of a phosphate-buffered 2 % solution of formaldehyde (pH 7.5), i.e. to give a final formaldehyde concentration of 0.2 %. After thorough mixing, a small amount of formalized culture was transferred to a Petroff-Hausser bacteria counter (chamber-depth 0.02 mm) and the leptospirae (N) in 20 small squares (each $\frac{1}{400}$ square mm) were counted at a magnification of 14×28 .

The number of leptospirae per ml formalized culture (N_f) was calculated by multiplying N by 10^6 (viz. $N/20 \times 400 \times 50 \times 1000$), and the number of leptospirae in the living culture (N_l) by multiplying N_f by $10/9$.

Countings were repeatedly made in duplicate or in triplicate. In the case of undiluted cultures the results of such repeated countings were mostly very consistent, the differences but exceptionally amounting to 15 or 22 % of the lowest figure found in a series. The results of repeated countings made from diluted cultures were more inconsistent, although the means of such countings corresponded quite well with the expected numbers of leptospirae in the dilutions. Therefore, in the tables the numbers of leptospirae in the culture dilutions are given as $\frac{1}{4}$ or $\frac{1}{8}$ of the calculated numbers of leptospirae in the undiluted cultures.

The average length (L) of the leptospirae in each culture was determined by measuring 25 leptospirae, chosen at random, with an eyepiece micrometer at the magnification 40×20 . In two instances this quantity was determined twice, with but small variations of the results (1.3 and 1.9 % of the lowest value).

Assuming an equal and constant thickness of all leptospirae the antigen density (D), in terms of the sum of the lengths of all leptospirae in one ml culture, was calculated by multiplying N_l by L.

Sera. - 12 sera were used, viz. 5 from human beings and 2*) from dogs with actual or previous leptospirosis, and 5 from rabbits, immunized by three to five 5-daily intravenous injections of increasing quantities of living c. one-week-old leptospira cultures (usually $1 \pm 2 \pm 5$ ml \pm , if necessary, 5 ml repeatedly).

Sera from human beings and dogs were native, while the rabbit sera were preserved by the addition of equal parts of sterile glycerine, or by addition of 1 % of a 1 % solution of sodium merthiolate.

Further details of the origin of the sera are given in table 1, part B.

Determination of Agglutinin-Lysin-titer. - From each serum a row of two-fold dilutions in saline was made with a Pasteur pipette (used throughout the whole experiment). 3 drops from each dilution were delivered into each of 6 dwarf test tubes, whereby 6 identical rows of dilutions were obtained. To each of the tubes in a row 3 drops of a living culture were added. Thereby it was possible to titrate each serum against 6 different cultures (or culture dilutions) simultaneously. As controls served (1) a mixture of equal parts of saline and culture (negative controls), and (2) a mixture of 3 parts of saline and 1 part of culture (50 % controls).

After having been well shaken the wire stand with the tubes was left at room temperature (c. 22° C.) for 4-6 hours, covered with a pad of paper tissue to counteract evaporation.

Reading was then made by examining in dark-field, at a magnification of 20×7 , a droplet from each tube, taken with a platinum loop and placed on a slide without coverslip.

*) These sera were obtained from the Danish State Veterinary Serum Laboratory, by courtesy of H. E. Ottosen, Vet. M.D.

Table 1.
Details of strains and sera.

A. Strains:

Name	Date isolated	Origin	Onset of patient's disease	Strain isolated from	Serological type of strain
M ₂₀	12/7 1935	man	24/6 1935	urine via cavia	icterohaemorrhagia
M ₆₃₅	13/11 1945	"	10/11 1945	blood directly	"
M ₈₁	5/11 1937	"	2/11 1937	"	sejroe
M ₈₀₉	31/10 1948	"	26/10 1948	"	"
H. Utrecht IV	Dec. 1931	dog	not known	urine via cavia	canicola
Alarich	c. 1932-1933	"	"	?	"

B. Sera:

No.	Date taken	Origin	Disease or immunization begun	Demonstration of leptospirae	Serological type of leptospiral infection
W. 2463	9/10 1948	man	24/6 1935	strain M ₂₀ isolated from urine	icterohaemorrhagia
W. 2269	27/9 1948	"	30/4 1948	" M ₇₉₈ " " blood	"
W. 2955	10/11 1948	"	26/10 1948	" M ₈₀₉ " " "	sejroe
W. 2786	31/10 1948	"	30/5 1944	leptospirae not demonstrated ¹⁾	"
W. 2386	4/10 1948	"	6/9 1948	" " " "	canicola
409 + 429 ³⁾	409: 9/11 1948 429: 19/11 1948	dog	c. 1/11 1948 not known	leptospirae not demonstrated ¹⁾ microscopically in urine (9/11-48)	canicola
415	12/11 1948	"	c. 1/10 1948	" " " " (25/10 48)	"
K. 9680 ⁵⁾	23/1 1946	rabbit	4/1 1946	immun. with strain M ₂₀	icterohaemorrhagia
K. 138 ⁵⁾	21/6 1947	"	28/5 1947	" " M ₆₉₅	"
K. 860 ⁶⁾	18/12 1937	"	30/11 1937	" " M ₈₁	sejroe
K. 123 ⁶⁾	5/2 1947	"	22/1 1947	" " M ₇₅₀	"
K. 3884 ⁶⁾	6/11 1941	"	17/10 1941	" " H. Utrecht IV	canicola

¹⁾ The diagnosis was substantiated by a rise of the serum titer with *L. sejroe* from 1:300 (7/6 44) to 1:3,000 (13/6 44).

²⁾ The diagnosis was substantiated by a serum titer of 1:10,000 with *L. canicola*.

³⁾ Mixture of two very small samples of serum.

⁴⁾ The diagnosis was substantiated by a serum titer of 1:3,000 with *L. canicola*.

⁵⁾ Preserved by sodium metriborate 0.01 %.

A reaction was considered positive when the number of free-lying leptospirae was nearer to the number of free-lying leptospirae in the 50% control than to that in the negative control, i. e. when $> 30\%$ of the leptospirae originally present were lysed, agglutinated or gathered in clumps of lively motile leptospirae, the so called 'breeding nests' of Schaffner & Mochtar (1927).

The serum titer was determined as the highest serum dilution (or the reciprocal value of the lowest serum concentration), which gives a positive reaction as defined above.

Experiments and Results.

Influence of the antigen density on the serum titer. - Three experiments, with strains of different serological types, were performed. In each experiment four sera of different origin were titrated simultaneously against two strains of the same serological type, cultures of each strain being used undiluted, diluted 1:1, and diluted 1:16. At the same time the number of leptospirae per ml in the cultures (N_1) and the average length (L) of the leptospirae in the different cultures were determined, and the antigen densities (D_m) in the mixtures of serum dilution and culture (respectively culture dilution) were calculated as $D_m = \frac{N_1 \times L}{2}$.

Table 2.

Results of agglutination-lysis-tests with various dilutions of cultures of two strains of *Leptospira icterohaemorrhagiae*.

Serum	5-day-culture of <i>L. icterohaemorrhagiae</i> in medium 555									
	strain Mo					strain Mo c				
	Dilution of culture	N_1 ($\times 10^6$)	L (μ)	D_m (m)	Titer	Dilution of culture	N_1 ($\times 10^6$)	L (μ)	D_m (m)	Titer
W 2163	1:1	217	10.1	1130	100	1:1	219	9.5	1040	200
	1:1	54	"	280	500	1:1	55	"	260	100
	1:16	11	"	70	1,600	1:16	11	"	65	800
W 2269	1:1	217	"	1130	6,000	1:1	219	"	1040	3,200
	1:1	54	"	280	12,800	1:1	55	"	260	6,400
	1:16	11	"	70	12,800	1:16	11	"	65	12,800
K. 9680	1:1	217	"	1130	12,800	1:1	219	"	1040	12,800
	1:1	54	"	280	25,600	1:1	55	"	260	25,600
	1:16	11	"	70	51,200	1:16	11	"	65	51,200
K. 438	1:1	217	"	1130	12,800	1:1	219	"	1040	12,800
	1:1	54	"	280	25,600	1:1	55	"	260	25,600
	1:16	11	"	70	51,200	1:16	11	"	65	25,600

N_1 = calculated number of leptospirae in 1 ml culture (or culture-dilution),

L = average length of leptospirae in the culture,

D_m = antigen density in the mixtures of serum dilution and culture (or culture dilution), calculated as $\frac{N_1 \times L}{2}$.

Table 3.

Results of agglutination-lysis-tests with various dilutions of cultures of two strains of *Leptospira sejroe*.

Serum	6-day-cultures of <i>L. sejroe</i> in medium 555									
	strain M84					strain M899				
	Dilution of culture	N ₁ (×10 ⁶)	L (μ)	D _m (m)	Titer	Dilution of culture	N ₁ (×10 ⁶)	L (μ)	D _m (m)	Titer
W. 2955	1:1	166	8.0	660	6,400	1:1	124	6.3	390	6,400
	1:4	42	»	165	12,800	1:4	31	»	98	6,400
	1:16	10	»	41	?	1:16	8	»	24	12,800
W. 2786	1:1	166	»	660	400	1:1	124	»	390	400
	1:4	42	»	165	800	1:4	31	»	98	800
	1:16	10	»	41	1,600	1:16	8	»	24	800
K. 860	1:1	166	»	660	3,200	1:1	124	»	390	6,400
	1:4	42	»	165	6,400	1:4	31	»	98	25,600
	1:16	10	»	41	12,800	1:16	8	»	24	51,200
K. 123	1:1	166	»	660	12,800	1:1	124	»	390	25,600
	1:4	42	»	165	25,600	1:4	31	»	98	51,200
	1:16	10	»	41	51,200	1:16	8	»	24	102,400

Explanation of abbreviations is given in table 2.

Table 4.

Results of agglutination-lysis-tests with various dilutions of cultures of two strains of *Leptospira canicola*.

Serum	7-day-cultures of <i>L. canicola</i> in medium 555									
	strain: H. Utrecht IV					strain: Alarich				
	Dilution of culture	N ₁ (×10 ⁶)	L (μ)	D _m (m)	Titer	Dilution of culture	N ₁ (×10 ⁶)	L (μ)	D _m (m)	Titer
W. 2386	1:1	233	7.7	900	3,200	1:1	177	8.8	780	12,800
	1:4	58	»	225	6,400	1:4	44	»	195	25,600
	1:16	15	»	56	12,800	1:16	11	»	49	25,600
K. 3884	1:1	233	»	900	6,400	1:1	177	»	780	6,400
	1:4	58	»	225	6,400	1:4	44	»	199	12,800
	1:16	15	»	56	12,800	1:16	11	»	49	25,600
409+429	1:1	233	»	900	25,600	1:1	177	»	780	51,200
	1:4	58	»	225	25,600	1:4	44	»	195	102,400
	1:16	15	»	56	51,200	1:16	11	»	49	102,400
415	1:1	233	»	900	6,400	1:1	177	»	780	12,800
	1:4	58	»	225	12,800	1:4	44	»	195	25,600
	1:16	15	»	56	12,800	1:16	11	»	49	51,200

Explanation of abbreviations is given in table 2.

The results, which are tabulated in tables 2, 3 and 4, show clearly that the titer of a serum against a given strain increases with decreasing antigen density, irrespective of the serological type of the culture, or of the animal origin of the serum used. In the discussion we shall return to the relationship between antigen density and serum titer.

It is also seen from these experiments, that the titer of a given serum with different strains of the same serological type is not always the same, despite the antigen density of the two strains being of the same magnitude. For instance, the strains M_{20} and Alarich seem to be slightly more »sensitive« than the strains $M_{60.5}$ and H. Utrecht IV, respectively.

Influence of the age of the culture on the serum titer. — To get an impression of the importance of the age of a culture for the serum titer, two experiments with strains of two different serological types were performed. In each experiment two sera were titrated simultaneously against 2 strains of the same serological type, using cultures of different age (the variation of the age, however, being kept within reasonable limits). The antigen densities of the cultures were determined as in the previous experiments.

The results of these experiments, which are tabulated in tables 5 and 6, show that when the antigen densities were of the same magnitude, increasing age of the culture in some cases caused a slight decrease of the serum titer, whereas in other cases the serum titer remained unchanged (see table 6, strain $M_{8.4}$). In the case of the strain $M_{8.0}$, (see table 6) a considerable decrease of the antigen density with increasing age complicated the evaluation of the results as regards the influence of the culture age as such. To this point, too, we shall return in the discussion.

Possible influence of the medium used for the cultivation of the antigen on the serum titer. — In the few experiments here presented no such influence was observed, when the culture age and the antigen density were of the same magnitude (see table 6, 14- and 15-day-cultures of $M_{8.4}$, also compare 7-day-culture of $M_{8.4}$ in table 6 with 6-day-culture in table 3, and 6-day-cultures of M_{20} and $M_{60.5}$ in table 5 with 5-day-cultures in table 2).

Discussion and Conclusions.

In the experiments recorded above it was found (1) that the titer of a serum with a given leptospira culture increased with decreasing antigen density.

If we assume that, at least within the range of antigen densities usually employed in the agglutination-lysis-test, the relation between the logarithm of the antigen density and the logarithm of the serum titer can be adequately expressed by a straight line (and nothing in

Table 5.
Results of agglutination-lysis-tests with cultures of different age.

26/11 48	Undiluted culture of <i>L. icterohaemorrhagiae</i>											
	strain: M20						strain M695					
	Age of culture (days)	Medium	Ni ($\times 10^6$)	L (μ)	Dm (m)	Titer	Age of culture (days)	Medium	Ni ($\times 10^6$)	L (μ)	Dm (m)	Titer
K. 9680	6	554	198	9.6	950	6,400	6	554	230	7.8	900	12,800
	13	"	225	9.1	1020	6,400	13	"	249	8.5	1060	6,400
	20	"	146	10.6	770	6,400	20	"	194	9.6	930	6,400
K. 138	6	554	198	9.6	950	12,800	6	"	230	7.8	900	12,800
	13	"	225	9.1	1020	12,800	13	"	249	8.5	1060	6,400
	20	"	146	10.6	770	6,400	20	"	194	9.6	930	6,400

Explanation of abbreviations is given in table 2.

Table 6.
Results of agglutination-lysis-tests with cultures of different age and, partly, grown in different batches of a modified Korthof's medium.

27/11 48	Undiluted culture of <i>L. sejroe</i>											
	strain: M84						strain: M809					
	Age of culture (days)	Medium	Ni ($\times 10^6$)	L (μ)	Dm (m)	Titer	Age of culture (days)	Medium	Ni ($\times 10^6$)	L (μ)	Dm (m)	Titer
W. 2055	7	554	188	9.9	930	12,800	9	555	117	6.7	390	12,800
	14	"	144	10.4	750	12,800	15	"	33	7.2	120	25,600
	21	"	127	11.0	700	12,800						
	15	555	143	10.2	730	12,800						
K. 860	7	551	188	9.9	930	3,200	9	555	117	6.7	390	6,400
	14	"	144	10.4	750	3,200	15	"	33	7.2	120	12,800
	21	"	127	11.0	700	3,200						
	15	555	143	10.2	730	3,200						

the results of the experiments hitherto performed speaks against this assumption) the results in tables 2, 3 and 4 show that *ceteris paribus* the serum titer is inversely proportional to a power of about 0.4 of the antigen density.*) Our use of the expression »about 0.4« will serve to emphasize that this approximate value is given with every reservation as to the outcome of further experiments. Only such experiments can decide whether or not the relation is the same for different sera and/or for different strains, and whether this relation(s) can be adequately expressed by a straight line.

The experiments also showed (2) that in some cases different strains of the same serological type gave different titers with the same serum, despite the employment of cultures grown in the same medium, of the same age, and of approximately the same density. This phenomenon of different »sensitivity« of serologically identical strains is well known by all leptospirologists, and is probably related to the phenomenon of varying »sensitivity« of a single strain at different times.

Furthermore it was found (3) that increasing age of a culture might influence the serum titer in various ways, as in some cases the titer showed a decrease, in others an increase, while in other cases again it remained unchanged with increasing culture age.

However, since a change in the density of a culture influences the serum titer, a possible influence of the culture age as such on the serum titer can only be evaluated after due consideration of the antigen densities of the cultures in question.

It is seen from tables 5 and 6 that after a certain period of time, varying according to the general »condition« of the strain and also dependant on the cultivation technique, the number of leptospirae in a culture decreased. Although as a rule this decrease in number was associated with an increase in the average length of the leptospirae, the latter but rarely counterbalanced the former; therefore, the antigen density of older cultures was generally less than that of young cultures. Consequently, on account of the lesser density of an old culture, one would expect this to give a higher serum titer than a corresponding young culture. Nevertheless, the serum titer in most cases remained unchanged or even decreased with increasing culture age, which phenomenon can hardly be explained otherwise than by a decreased »sensitivity« of the leptospirae in the older cultures.

In the case of the strain M_{809} which (being a quite recently isolated

*) All the observations being treated as a whole, \log_2 serum titer was plotted against \log_2 antigen density, the lowest \log_2 antigen density and the highest \log_2 serum titer in each triplet of observations being called 0. The means (m_0 , m_2 and m_4) of \log_2 serum titer's for each \log_2 antigen density (0, 2 and 4) having been calculated, a straight line was drawn through m_0 and m_4 . The slope (b) of this line ($\log y = b \times \log x$) was -0.41 . It should be added that the point m_2 was situated quite near to the line $m_0 m_4$.

strain) showed a considerable decrease in antigen density from the 9th to the 15th day, the increase in serum titer with the older culture of this strain may be considered roughly equivalent to the decrease in antigen density.

Finally, (4) no influence on the serum titer was observed by the use of different batches of medium for the cultivation of the antigen employed in the agglutination-lysis-test. However, in relation to this point we want to recall the observations of *Lumley* (1937) and *Malmgren* (1941), that the »sensitivity« of a given leptospira strain may vary considerably from time to time. Both *Lumley* and *Malmgren* were of the opinion that this variation was probably due to some varying factor in the medium.

In view of the experiences mentioned above, as well as of similar experiences previously made at the Danish State Serum Institute, we feel justified in concluding that the results of agglutination-lysis-tests, performed in different laboratories (or in the same laboratory at different times) are not necessarily directly comparable, even though the same strain and the same technique (including end-point determination) are employed, unless the following factors are also taken into consideration (a) the »sensitivity«, (b) the age, and (c) the density of the cultures used for the test.

However, in all probability it should be possible to overcome, with relative ease, the difficulties arising from variations of the factors considered here.

(a) A standardization of the »sensitivity« of different cultures could be obtained by the titration (using a standardized technique) of these cultures against standard-sera, kept under optimal conditions. In the case of formalized cultures this method has already been used successfully by *Gardner* (1943). A suitable correction could perhaps then be introduced for such variations in the serum titer which are caused by variations in the sensitivity of a given strain.

(b) The influence of the culture age factor will probably be negligible, if the cultures are used only when the number of leptospirae is at or near its peak.

(c) The density of the cultures could either be standardized, or a suitable correction be introduced, when the relationship between the antigen density and the serum titer has been more exactly determined.

It is true that provided the sensitivity of a formalized culture, kept under optimal conditions, is constant, the employment of such cultures would mean a considerable simplification of the standardization work. *Gardner* (1943) controlled the agglutinability of one batch of formalized culture (0.25 % formalin) for more than two years, during which time it showed scarcely any significant decrease. According to *Zuelzer* (1936) leptospira cultures, formalized by the addition of 0.1—1 % of a 35 % formaldehyde solution and kept in sealed dark

glass-tubes, were still »durchaus zuverlässig« after 6 months, but their actual sensitivity does not seem to have been controlled. Many laboratories employ formolized cultures with confidence, apparently without actually controlling their sensitivity. On the other hand, some laboratories still experience difficulties in the preparation of reliable formolized leptospira suspensions. It is possible, however, that the employment of a protein-free suspension-medium, such as that used by Pot (1936), may solve this problem.

It should, therefore, be investigated whether the conclusions arrived at above in regard to the agglutination-lysis-reaction are valid also for the agglutination-reaction with formolized leptospira suspensions.

One may ask whether the whole matter is worth the effort. We believe it to be of some importance for the following reasons:

(1) A standardization of the agglutination-lysis-test (respectively the agglutination-test with formolized cultures) after the principles outlined above, would make possible a direct comparison of the results obtained in different laboratories, or in the same laboratory at different times.

Experiences gained in one laboratory could then be utilized directly by other laboratories which use the same standardized test. As an example *Esseveld* (1937), summarizing the extensive experiences gained in *Schöffner's* laboratory in Amsterdam, finds that a serum titer of 1:300 may generally be considered a sign of actual or previous leptospirosis. These experiences, being gained in a single laboratory, are presumably based on tests performed with cultures of a fairly uniform density. If we assume this to be equivalent to $100-200 \times 10^6$ leptospirae per ml, it is evident that titers found in another laboratory (Y), using cultures with for instance 10×10^6 leptospirae per ml, are not directly comparable with the Amsterdam titers and, consequently, the experiences from Amsterdam cannot be utilized directly by Y-laboratory. Provided the technique used in Y-laboratory is otherwise the same as in Amsterdam, a Y-titer of 1:300 may be roughly equivalent to an Amsterdam-titer of 1:100 only, while an Amsterdam-titer of 1:300 may be equivalent to a Y-titer of about 1:900.

(2) When a serum from a patient reacts with strains of different serological types, one generally assumes that the infection of the patient is caused by a leptospira of the same serological type as the strain which gives the highest titer (provided the test is performed with strains of all the serological types which cause leptospiroses in the population in question, and provided the serum sample is taken sufficiently late in the disease, i. e., later than the 4th week). Here, too, it is necessary to take into account the titer-influencing factors mentioned above. For instance, if the density of a culture of type P-leptospirae is about 30 times the density of a culture of type Q-leptospirae, the infection of a patient may quite well have been caused by

a leptospira of type P, even though the titer of the patient's serum with type Q-culture is double that with type P-culture. If a Q-culture of the same density as the P-culture had been used, the Q-titer would have been only half the P-titer.

(3) Also in other cases for the true estimation of the strength of co-agglutinations it is necessary to consider the antigen densities and other factors influencing the serum titers.

It will be understood from the above discussion that if the differences in antigen density, culture age, and culture »sensitivity« are only small, the influence of these factors on the serum titer is negligible, whereas, on the other hand, major differences in these quantities may cause considerable differences in the serum titer, especially if they are working in the same direction. Therefore, the importance of these factors should not be entirely overlooked.

Summary.

1. Experiments are recorded which show that *ceteris paribus* the titer of a serum, found by the agglutination-lysis-test with living leptospira cultures, increases with decreasing antigen density. The relation between antigen density and serum titer is preliminarily determined.

2. Increasing age of the culture seems to influence the serum titer in two ways. Apparently the »sensitivity« of the leptospirae decreases with increasing age and for this reason the serum titer would decrease. However, a decrease in antigen density with increasing culture age may counterbalance, or even overcompensate, the decrease in »sensitivity«.

3. No influence on the serum titer was observed in our experiments by the use of different batches of culture medium. However, according to other workers the serum titer may be influenced considerably by variations in the »sensitivity« of a given leptospira culture, probably due to factors in the culture medium.

4. The possibility and the importance of a standardization of the agglutination-lysis-test (and of the agglutination-test with formalized cultures) are discussed.

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BETA-HAEMOLYTIC STREPTOCOCCI IN THROAT AND ANTISTREPTOLYSIN TITRE

By Thorolf Packalén.

(Received for publication February 2nd, 1949.)

An elevated antistreptolysin (AS) titre in serum is as a rule due to the presence of antibodies specifically formed against streptolysin-O-producing streptococci. Non-specific increases are rare, having been observed e. g. in sera from hepatitis patients (5, 12, 18, 23), and in sera contaminated with certain bacteria (3, 8, 12).

Everybody comes from time to time into contact with the ubiquitous β -streptococcal agent, which results in a more or less marked rise in the AS titre. When the infection (manifest or latent), which follows the contact, subsides, the titre as a rule declines. The antibody level often remains slightly higher than prior to the contact, however. Not infrequently such a moderately raised antibody level is the only sign of persisting dormant streptococcal foci. The majority of investigators have found the borderline value of "normal" AS titres to be approximately 200 units. Percentages of titres exceeding that value in "healthy" individuals have varied between 0 and 26 per cent (cf. table 2 in 7). When subjected to a closer clinical and bacteriological examination, however, many "healthy" persons in such groups have been found actually or very probably to be infected with haemolytic streptococci (6, 7).

The most important portals of entry for streptococcal infections are the tonsils and the fauces. These are at the same time the sites where probably the majority of streptococcal carriers harbour their germs. The streptococcal carrier rate in human beings varies of course greatly in different climates, seasons, epidemiological environments, and at various ages.

Modern enrichment techniques have increased the rates of identified carriers to up to 30—40 per cent (13—15).

In a bacteriological and serological study of 437 tuberculous and medical patients for multiple infection the throat flora was examined, inter alia for the presence of β -haemolytic streptococci, and the blood serum for its AS titre. After deducing from the total material 51 patients showing definite clinical symptoms of streptococcal disease, i. e. tonsillitis, rheumatic fever, acute glomerulonephritis, septic infections, there emerged a carrier rate of 53 per cent. This is a high percentage, but it is to be stressed that the majority of the patients were tuberculous cases. The significance of the high proportion of streptococcal carriers in such a material will be discussed in another paper.

However, in addition to this the material collected invited to an analysis of the relationship between the carriage of β -haemolytic streptococci in the throat, and the AS level.

Material.

Of the persons examined 337 had tuberculosis, the majority of them pulmonary tuberculosis, several had pleurisy, and a few erythema nodosum tuberculous in type. The remaining 100 cases consisted of patients with various internal diseases.

As stated above 51 patients had "genuine" streptococcal diseases. Many of the remaining 386 patients suffered of conditions, such as pneumonia, bronchopneumonia, bronchiectasis, bronchial asthma, pharyngitis, etc., which as a rule are not streptococcal in origin, but haemolytic streptococci may sometimes play a more or less preponderant role in them. Because of the familiar difficulty or in many instances even impossibility to differentiate these complications from the tuberculous processes proper no attempt has been made, however, to subdivide the patients into groups according to the presence or absence of such potentially streptococcal conditions.

Procedure.

Throat samples for bacteriological examination were taken by swabbing the tonsils and fauces. The majority of patients were examined repeatedly with one or several weeks' intervals: two thirds at least twice, one third at least thrice, some of them up to 8—10 times. The swabs were rubbed into two 5 per cent horse blood agar plates. One was incubated aerobically the other anaerobically. Beta-haemolytic colonies were identified by microscopy, and titrated for production of soluble haemolysin. To one ml of serial dilutions of a 24^{hrs} 0.2 per cent glucose infusion broth culture was added 0.5 ml of a 3 per cent suspension of washed sheep blood corpuscles, and the mixture incubated at 37° C. for one hour. The 50 per cent haemolysis end point was read.

Not infrequently strains were encountered which only irregularly and slowly gave a positive haemolysin test. Therefore typical β -haemolytic streptococci were not regarded as lacking the capacity to form soluble haemolysin before retested several times with uniformly negative results.

A number of strains were tested with Lanecfield's grouping sera, and for hyaluronidase production with the mucin-clot-prevention test (M. C. P.) according to McClean (10).

The antistreptolysin test was in all essentials performed according to Ipsens technique (4). Sera were inactivated at 56° C. for half an hour, and preserved frozen at about -25° C., when not immediately examined. Todd's immune horse serum was used as a standard, or patients' sera, the titres of which had been previously estimated by repeated determinations against Todd's serum.

The reading of the titre end-point deviated from Ipsen's procedure in that the 50 per cent haemolysis limit was used. For details the reader is referred to a paper on antistaphylococcal titrations (13).

Results.

Carrier rates. Out of 386 patients without definite clinical signs of streptococcal infection 204, or 52.8 per cent, had β -haemolytic streptococci in one or several of their throat swabs. Of 51 patients with such symptoms 43, or 84.4 per cent, yielded positive throat cultures. It should be stressed, however, that the percentages recorded do not represent the true carrier rates, but only minimum values, as will be apparent from Table 1.

Table 1.

Significance of repeated swabbing for identification of maximum number of streptococcal carriers.

No. of swabs examined	Patients examined	Patients with β -streptococci in their throat cultures	
		No.	per cent
1	437	175	40.1
2	294	159	54.1
3	136	99	72.7
4	80	54	67.5
5	44	31	70.5
6	30	21	70.0

This shows, namely, the importance of repeated throat cultures for obtaining maximum results. A single examination and even two consecutive swabs seem to miss a certain number of actual carriers, which are identified by three (or more) examinations.

Incidentally this observation obviously corroborates the correctness of the public health authorities' usual demand of three consecutive negative swabs before declaring a person free of infection.

Haemolytic power of throat streptococci and manifestation of streptococcal disease. Strains isolated from 163 of the patients without clinical signs of streptococcal infection and from 39 of those having such symptoms were tested for production of soluble haemolysin. Strains from 121 of the former and 34 of the latter patients were examined

as to their serological group (Lancefield). When strains isolated on different occasions from the same patients, or when the same strain on repeated examinations gave varying haemolysin titres, the highest ones observed were considered as representative of the patients and the strains respectively.

Table 2.

The incidence of manifest streptococcal disease among carriers of β -streptococci with varying capacity to form soluble haemolysin.

Haemolysin titre of isolated streptococci	Patients carrying β -streptococci		
	Totals	Showing clinical signs of streptococcal disease	
		No.	per cent
0	42	2	4.8
1/1	9	1	11.1
1/4	16	2	12.5
1/16	49	6	12.2
1/64	51	13	24.1
1/256	32	15	46.9
(Non-carriers	190	8	4.2)

Table 2 shows clearly that strains with strong lysin production were, relatively taken, considerably more frequent among patients with clinical streptococcal disease than among streptococcal carriers without symptoms of such a condition. Thus, 24 and 47 per cent respectively of all the patients harbouring β -streptococci capable of producing soluble haemolysin up to titres of 1/64 and 1/256 or higher, suffered from clinically established streptococcal infection, as against only 5 to 12 per cent of patients carrying β -streptococci without any lysin formation or producing lysin to a low titre only, viz. 1/1 — 1/16. For comparison may be stated that for patients with cultures negative for β -streptococci the corresponding percentage was 4.2.

Haemolytic power of throat streptococci and AS level in serum.
A great number of investigators have shown that manifest streptococcal infections, tonsillitis, scarlet fever, rheumatic fever, acute glomerulo-nephritis, etc. are accompanied by a more or less marked rise in the antistreptolysin titre, viz. between 70 and 100 per cent of the cases. In the present group of 51 patients the percentage of "elevated" AS titres (200 units or more) was 76.5.

The high rate, 52.8 per cent, of streptococcal carriers observed in the present material of patients not displaying definite symptoms of streptococcal disease corresponded to a proportionally increased incidence, 45.7 per cent, of elevated AS titres. It seemed of considerable interest to analyze whether there existed also a quantitative rela-

tionship between the AS level in the serum and the power of haemolysin production of the streptococcal strains in the throat.

From the graphs in Fig. 1 it will be seen that such a correlation did exist. Patients carrying in their throat strains capable of an ample haemolysin production (titres 1/64 and over) had a higher rate of elevated AS titres than those carrying weak haemolysin producers

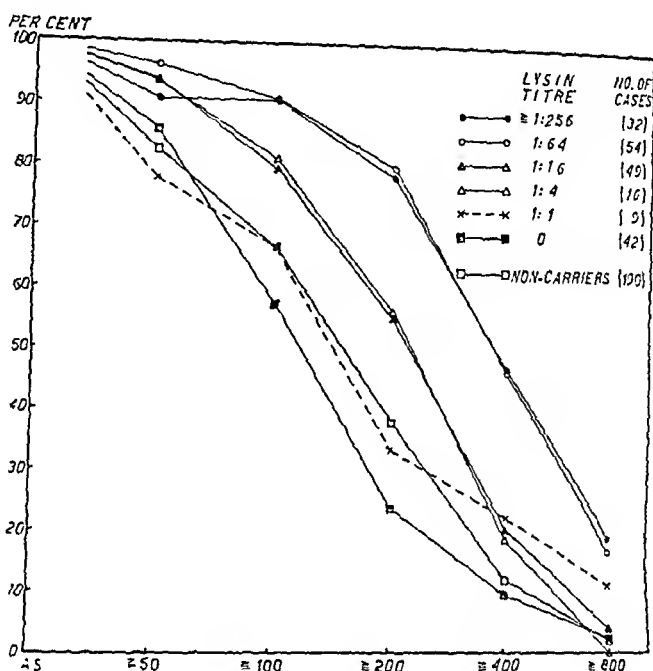


Figure 1.

Relationship between haemolytic power of throat streptococci and AS level (expressed by percentages of streptococcal carriers having AS titres equal to or exceeding 50, 100, 200, 400 and 800 I. U., respectively).

(titres 1/4—1/16). Lowest was the incidence of raised AS titres among carriers of haemolysin-negative β -streptococci and non-carriers. Patients carrying the weakest haemolysin-producers (titre 1/1) were in this material too few in number (9) to be considered representative of their group. Their correlation curve suggests, however, that so weak a haemolysin production may be inadequate as a stimulant of antibody formation.

The graphs in Figure 1 refer to all streptococcal carriers under investigation, i. e. irrespective of clinical symptoms of streptococcal disease. However, a deduction of all the patients with such symptoms from the total material, does not change essentially the results recorded.

Carriage in the throat of β -streptococci belonging to different Lancefield's groups and AS level in serum. Streptococci producing strepto-

lysin O, which is the antigen responsible for the specific rises in AS titres, belong all to Lancefield's serological groups A, C or G. The soluble haemolysins sometimes produced by β -streptococci belonging to Lancefield's other groups, B, E, F, L, M are antigenically unrelated to streptolysin O. Therefore, the incidence of elevated AS titres should be expected to be higher among carriers of β -streptococci belonging

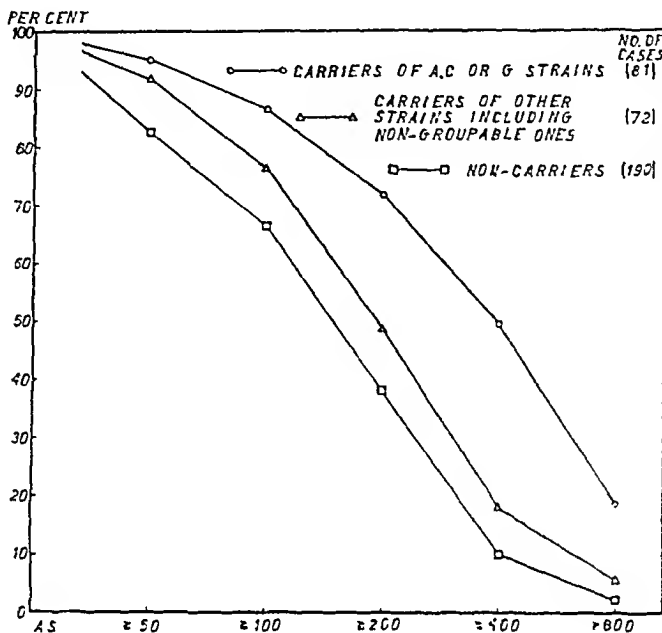


Figure 2.

Relationship between carriage of β -streptococci belonging to Lancefield's groups A, C and to other groups respectively, and AS level in serum (expressed as in Fig. 1).

to the three first-mentioned groups than among other carriers. Fig. 2 shows that this actually was the case.

For comparison the curve of non-carriers is inserted in the graph. It will be noted that its course is on a somewhat lower level than the curve representing carriers of β -streptococci other than those belonging to groups A, C or G. The last-mentioned curve, however, comprises not only (36) carriers of β -streptococci identified as belonging to Lancefield's other groups (than A, C or G) but also 35 patients carrying β -streptococci the grouping of which has been unsuccessful owing to technical reasons. It may be assumed that a number of these lastmentioned strains have actually belonged to groups A, C or G, a circumstance which would explain the observed slight elevation of the curve for this group of carriers.

This view is supported by the results of an examination of the

isolated β -streptococci for hyaluronidase production. The McClean mucin-clot-prevention (M. C. P.) test was used (10). So far as present information goes predominantly strains belonging to Lancefield's groups C (human type) and G are capable of producing hyaluronidase. Of group A strains only those belonging to Griffith's types 4 and 22 have been observed to form the enzyme. In the present material none of 72 A group strains gave a positive M. C. P. test, whereas 35 of 41 C strains and 31 of 34 G strains did so. Of 53 "non-groupable" strains subjected to the M. C. P. test 10 were found to be positive. This makes it probable that at least some C and G strains actually were masked among the "non-groupable" strains. It seems justified by analogy to assume that a proportional number of M. C. P.-negative A group strains also may have been masked in this category.

Persistency of streptococcal carriage and stability of antistreptolysin level. When occasionally negative swabs obviously due to technical imperfection in the isolation of the micro-organisms are disregarded, the outstanding feature in the findings is the permanency of the carrier state over long periods of observation. Of 70 carriers followed for more than 3 months only 15 eventually gave a negative culture. It should be noted, moreover, that in 10 of them the negativity referred to a single swab, and consequently may mean merely a failure of the isolation technique rather than actual disappearance of the streptococci.

The AS titres recorded in the carriers are in good keeping with this observation of the persistency of their carrier state. Of 181 carriers followed with two or more throat swabs only 25, or 13.8 per cent. showed an increase in their AS values by more than 100 per cent (or decrease by more than 50 per cent). In assessing this experience, all the titres recorded from one month before the first swab until 3 months after the last one have been taken into account. Of 32 patients displaying clinical signs of streptococcal disease six, or 18.8 per cent, showed titre changes as against 19, or 12.8 per cent, of 149 patients without such symptoms. For comparison may be recorded that among 113 non-carriers the corresponding percentage was 3.5.

Finally it should be stated that in 13 cases the rise or fall in titre coincided within reasonable time limits with the appearance or disappearance of β -streptococci in the throat flora, in the remaining 12 cases failed to do so.

Comment.

The haemolysin producing power of β -streptococcal strains isolated from the throats of patients displaying clinical symptoms of streptococcal disease was on an average stronger than of those from streptococcal carriers. Rather than indicating that strong haemolysin produc-

tion must elicit clinical signs of streptococcal disease — in the present series not less than 67.5 per cent of all strains having a lysin titre of 1/64 or higher derived from streptococcal carriers without such signs — the observation seems only to show that strains with weak haemolysin formation seldom cause manifest symptoms.

This tallies with the observation that carriers of β -streptococci with no or very weak lysin production do not show any elevated antistreptolysin levels as compared with non-carriers. On the other hand, carriers even of moderately lysin-active strains (titres 1/4—1/16) showed a considerable rise in their AS levels, although comparatively few of them displayed clinical signs of streptococcal disease. A more marked rise in AS titres was seen in patients harbouring strong haemolysin producers. This held true even when all patients with signs of manifest streptococcal disease were excluded from the compiled material.

The possibility has to be considered that in a number of carriers the tonsillar and faecal mucosa may be the only site of the infecting streptococci. An elevated AS in such individuals were then elicited by haemolysin absorbed from the surface of the mucosa. On the other hand, considerable evidence has been presented for this not being the sole event. Adamson (1, 2) and Ringertz and Adamson (17) using necropsy material have shown in comprehensive bacteriological, serological and histo-pathological studies of the lymph nodes of the respiratory tract that haemolytic streptococci not infrequently invaded these glands and even elicited in them a more or less characteristic cellular response. Furthermore the probable synergism of tubercle bacilli and streptococci (1, 2, 9, 11, 12, 19—22) observed in many tuberculous subjects all but presupposes a more intimate contact between host and germ than is likely to occur on the surface of the mucous membranes of the nasopharynx. Also the long duration of the carrier state and the persistently raised AS level in many of them are easier to explain by assuming the existence of foci deeper than the nasopharyngeal ones.

Summary.

Beta-haemolytic streptococci were demonstrated in the throat cultures in 84.4 per cent of 51 patients suffering from infections commonly accepted as being of streptococcal origin. The streptococcal carrier rate in 386 (mostly tuberculous) patients without definite clinical signs of such an infection was 52.8 per cent.

A single or even two consecutive throat swabs seemed to miss a certain percentage of carriers, whereas three or more were effective.

Beta-streptococcal strains capable of ample haemolysin formation were more apt to elicit manifest symptoms of streptococcal disease than those producing little of the lysin or none.

Carriers of strains capable of ample haemolysin formation on an average had higher AS titres than those carrying weak lysin producers or strains devoid of any lysin formation.

In good keeping with this statement was the observation that patients carrying β -streptococci belonging to the streptolysin-O-producing groups A, C or G on an average had higher AS titres than carriers of other strains.

The β -streptococcal carriage, once established, was found to be surprisingly persistent, as were the elevated AS titres in the carriers.

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MOLONEY TEST AND ITS APPLICATION IN VACCINATION OF ADULTS

By I. Scheibel, S. Tulinius and K. Bojlén.

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In 1925, Zoeller (39) described a skin reaction, »anatoxi-reaction«, after i. e. injection of 0.2 ml. of a 1:100 dilution of diphtheria toxoid. Positive reaction, consisting of redness and infiltration on the site of injection, appeared in the course of one to three days, after which it faded and disappeared. He thought positive reaction was due to hypersensitivity to the protein of C. diphtheriae, and proposed that this reaction, together with the Schick reaction, could be used as a measure of the state of immunity against diphtheria, on the assumption that a Schick-positive person, who also showed positive anatoxin reaction, was on the way to spontaneous immunity. Moloney & Fraser in 1927 (22) noticed a correlation between anatoxin reaction and post-vaccinal reaction. They therefore suggested the use of this test as control for the Schick test, instead of the heated toxin dilution hitherto employed.

In succeeding investigations, Moloney & Fraser (23—24—8) made closer examination of the relation between skin test and local and constitutional reaction after immunisation. They proposed as standard dilution for the skin test that dilution of toxoid which produced 10—20 mm of redness in 50 % of persons between 20 and 30 years with positive skin reaction. According to our knowledge, however, this standard has not been followed, and as far as can be seen from the literature, 0.1 ml of a dilution 1:100 of the toxoid in question has been the dose most frequently used. The above-mentioned authors showed too that persons with strong positive skin reactions could be immunised with minute doses of prophylactics without getting post-vaccinal reactions.

The test was soon afterwards used as a routine test in mass-immunisation of children in Canada (6—7—19—20).

O'Brien & Parish (25), McSweeney (21) and Underwood (37) confirmed and elaborated these investigations. The test has been referred to in England as the »Moloney Test«. This name, which is now commonly adopted, will also be used in the present work.

In this country, Stig Thomsen (35) in 1944 stated in a short article that it was possible, by means of a similar test, to anticipate to a certain degree the post-vaccinal reaction of patients. He immunised positive persons with small doses and reported good correlation between skin reaction and post-vaccinal reaction.

Until recently, only children have been included in routine-vaccination in Denmark. The number of children with symptoms after injection of prophylactics has been very small, and the symptoms have been so negligible that we thought it unreasonable to bother the children with still another injection and to complicate field work for the doctors by the introduction of the Moloney test.

However, increasing morbidity amongst adults, even amongst the older age-groups, made it necessary in 1943—44 to start extensive immunisation of adults. It is a well-known fact that symptoms occur with greater frequency and severity in adults. We therefore considered it essential, as part of our endeavours to reduce these symptoms, to investigate more closely the advantages of the Moloney reaction.

The present investigation comprises 835 persons, aged 20—74 years, members of the staff of the Carlsberg Breweries, Copenhagen.*)

Moloney Test.

Technique.

0.1 ml of a dilution 1:100, corresponding to 0.05 Lf of the same toxoid, which after adsorption to $Al(OH)_3$ was used for the immunisation, was injected i. c. on the flexor side of the right forearm. The dilution was made with a phosphate buffer of pH 7.4. The reading was made 48 hours later. Reactions were classified according to the definitions given by Underwood (36): no reaction (\div); faint reaction (+) — definite area of erythema of less than 10 mm in diameter without induration, or any area of a faint pink colour which showed no thickening and which was not due to trauma; mild reaction (++) — an area of erythema of more than 10 mm in diameter,

Table 1.

Moloney results	No.	%
\div	380	45.5
+	77*)	9.2
++	107	12.8
+++	271	32.4
Total	835	

*) Three of this group later showed delayed reaction.

*) We are glad to take this opportunity of thanking Mr. Frederik Sander (at that time Managing Director of the Breweries), whose kindness and understanding made this investigation practicable, and also the workers and employees who, through their readiness and patience, contributed to its accomplishment.

with an area of slight induration; definite reaction (+++) — a large area of erythema up to 40 mm or more in diameter with a definite palpable area of induration in the centre.

The frequency of the different reactions is given in table 1.

The different reactions seem roughly to fall into similar size categories to those for adults in America and England, though the investigations carried out there have been chiefly on younger groups, as far as is known, not over 35 years of age. Moloney et al. (24) report 49 % and 24 % ++ and +++ reactions amongst 560 and 586 persons in the 20–35 and 10–19 age-groups respectively. O'Brien & Parish (25) found 29.4 % mild and definite reactions among 102 persons in the 19–28 age-group, Underwood (37) 35 % among 107 persons in the 15–19 age-group, and Brandon & Fraser (4) 35 % among 87 medical students. Bunch, Morrow, Timmons & Smith (5) report frequencies varying from 19–63 % in three groups of Schick-positive medical students and nurses.

In the present material, very extensive and unpleasant reactions were found in 48 out of 271 persons (17.7 %) in the +++ group, i. e. redness and oedema up the size of the palm of the hand, with a centre area showing bulla formation and in some cases slight necrosis of the tissue beneath. The reactions disappeared in the course of 4 to 9 days. Three cases were accompanied by rise in temperature to 40°, acute feeling of malaise and disability for work lasting for a few days. These strong reactions occurred with equal frequency in both sexes, independent of the patient's natural antitoxin titre and previous diphtheria. Moloney & Fraser (23) describe similar strong reactions in adults, but make no mention of bulla formation.

Influence of age, sex and history of diphtheria on the incidence of Moloney reactions.

Table 2.

Distribution of Moloney reactions according to age, sex and previous diphtheria.

Moloney results	+ Previous diphtheria								÷ Previous diphtheria							
	< 50 yrs.				> 50 yrs.				< 50 yrs.				> 50 yrs.			
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
÷	7	35	6	23	2	11	6	85	76	50.4	170	43.7	33	44.6	80	53.3
+	2	10	1	3	2	11	0	0	14	9.3	34	8.7	8	10.8	16	10.7
++	3	15	3	11	5	28	1	14	17	11.3	42	10.8	14	18.9	22	14.7
+++	8	40	16	61	9	50	0	0	44	29.2	143	36.8	19	25.7	32	21.4
Total	20		26		18		7		151		389		74		150	

Age.

A more detailed age analysis had to be abandoned because of the relatively few persons in the younger age-groups (a total of 32 under 30 years of age). We have therefore limited ourselves to two age-groups — under and over 50 years. It should be mentioned that a

trial distribution into further age-groups did not show any rise in Moloney positivity with advance in age among persons under 50 years.

Because of the greater number of persons it comprises, the group without previous diphtheria is the most suitable for an analysis of the effect of age and sex. It can be seen that the difference in frequency between the two age-groups is insignificant within all four reaction grades for women, and the same applies to men, except for the +++ group, where there seem to be fewer strong reactions in the group over 50 than under 50 years. A χ^2 calculation for comparison of the distribution shows that age is a relevant factor for men ($P = 1\%$) but not for women ($P = 40-50\%$).

Moloney et al. (24), McKinnon & Ross (20) and Underwood (37), whose investigations have chiefly comprised children and young people, found an increasing number of Moloney-positive reactions with advancing age. McKinnon & Ross (20) suggest that age in itself may influence the development of sensitivity, whereas Underwood considers that increased frequency of Moloney-positive reactions results from increased possibility of contact with *C. diphtheria*. As mentioned, we have not been able to demonstrate any increase in Moloney-positive reactions with advancing age, which rather supports Underwood's theory, since the age-groups dealt with here may be regarded as having equal possibilities for latent infection.

On the other hand, it is possible that the reduction in sensitivity in older age-groups revealed by our figures is a result of age, or possibly a combination of age and sex. However, our material does not furnish any further detail on this subject.

Sex.

A χ^2 comparison between the distribution of the various Moloney reactions in men and women shows that these do not differ significantly in either of the two age-groups. Sex, therefore, in agreement with Underwood's observations (37), does not seem to influence Moloney reactions.

History of Diphtheria.

Zoeller (39) observed that the number of »anatoxin reactions« rose during a diphtheria epidemic. Allin, Fraser & Hannah (1) demonstrated that patients may — and as a rule do — become Moloney-positive within a short period after an attack of clinical diphtheria. We have therefore investigated the question if persons with a history of diphtheria differ with regard to Moloney reaction from those without previous clinical diphtheria. The left half of Table 2 shows the distribution in this group. Direct comparison between the corresponding groups in the other half of Table 2 shows that most Moloney reactions are to be found among persons with previous diphtheria. As the groups are too small to permit a χ^2 calculation, we have limited our-

selves to a statistical evaluation of the difference between the relative frequencies of +++ reactions. This gives the following P values for the four possible comparisons: 32, 1, 6 and 63 %. The probability of obtaining such a series of percentages — assuming that the + and ÷ diphtheria groups are equal — is 4 %, which means that just significantly more positive reactions occur in the diphtheria group.

It should be mentioned that a decrease in strong reactions in men over 50 years of age can also be seen in the diphtheria group.

Relation between antitoxin content and Moloney reactions.

At the same time as the Moloney test was read, a blood-test was taken from all ++ and +++ reactors. The antitoxin content of this blood-test or the specimen itself will, in the following, be called AT_0 . It would have been desirable to investigate AT_0 in + and ÷ reactors as well, but it was impossible at that time, from a practical point of view, to take and titrate more blood-tests. Titration is made by intracutaneous injection after Claus Jensen's method (14).

No account has been taken of age, sex or history of diphtheria, as preliminary investigations showed that these factors did not appear to influence AT_0 within the age-groups in question. So far, this is in agreement with previous observations (13—33), as the age-group (under 30 years) which has been shown to have less natural immunity is so sparsely represented in the present material that it evades examination.

The results are shown in table 3.

Table 3.
 AT_0 and Moloney reactions.

Units per ml	Totals	Moloney results			
		++		+++	
		No.	%	No.	%
< 0.001	54	16	15.0	38	14.1
0.001—0.01	28	12	11.2	16	5.9
0.01—0.1	118	38	35.5	80	29.7
0.1—1	145	36	33.6	109	40.5
> 1	31	5	4.7	26	9.7
	376	107		269	

The percentages show directly that no difference is demonstrable between the frequency of persons without measurable AT_0 titre in the two Moloney groups. Among those with measurable titre, there seems to be a tendency towards more persons with higher values in the +++ group. The difference in frequency of titres over 0.1 AU in the two groups is just significant ($P = 5\%$).

Underwood (37), who also examined the distribution of AT_0 within Moloney ++ and +++ groups, finds 21.8 % (5/23) without measurable antitoxin among ++ reactors, as against 11.8 % (2/7) among +++ reactors, and 73.9 % (17/22) > 0.1 AU among ++ reactors as against 76.5 % (13/17) among +++ reactors. His groups being rather small, no significance is demonstrable.

Being unable to compare the natural immunity estimated from AT_0 titres in the \div and + groups on the one hand, with the two strongly positive groups on the other, we have examined whether the distribution of AT_0 in the latter is distinct from that in a group of persons who were not Moloney tested but whose AT_0 titres were determined a few months earlier.

The last-mentioned group contained a significantly larger percentage of persons under 30 years of age without measurable antitoxin titre, and this age-group has, therefore, been excluded to ensure better comparison.

The distribution of AT titres is seen in table 4.

Table 4.
 AT_0 in non-Moloney tested group.

Units per ml	No.	%
< 0.001	236	39.2
$0.001-0.01$	82	13.6
$0.01-0.1$	161	26.8
$0.1-1$	107	17.8
> 1	15	2.5
Total	601	

Comparison of this table with table 3 shows an obvious difference in the distribution. There are 2—3 times as many persons without measurable antitoxin titre in the non-Moloney tested group as in the Moloney-positive groups, while the frequency of persons with AT_0 over 0.1 AU is 1.9 and 2.5 times respectively greater in these two groups than in the non-Moloney tested group.

The differences are highly significant, the biggest P being under 1% . Though positive Moloney reaction is far from being synonymous with natural immunity — 20—26 % are under the Schick level (0.01 AU) — there is no doubt that the state of immunity, judged from AT_0 , is better in a Moloney-positive group than in a non-Moloney tested group.

This is in agreement with the findings of other authors (Zoeller (39); Moloney & Fraser (22); McKinnon & Ross (20); Underwood (37), Brandon & Fraser (4)).

Bunch, et al. (5) Moloney tested a group of Schick-positive persons and accordingly found a large majority of Moloney-negative or weakly positive reactions ($67/92 = 72.8\%$).

Plan of Immunisation.

Moloney results	AT ₀	Dose and time of injection				Total no. of inj.
		1st day	10th day	28th day	1 year	
÷	not measured	25 units (1.0 ml)		25 units (1.0 ml)	25 units (1.0 ml)	3
+	,	25 „ (1.0 ml)		25 „ (1.0 ml)	25 „ (1.0 ml)	3
-++	{ < 0.01	5 „ (0.2 ml)	5 units (0.2 ml)	5 „ (0.2 ml)	5 „ (0.2 ml)	4
	{ > 0.01	5 „ (0.2 ml)				1
+++	{ < 0.01	1 „ (0.5 ml, dil.)	1 „ (0.5 ml)	1 „ (0.5 ml, dil.)	1 „ (0.5 ml dil.)	4
	{ > 0.01	not immunised				0

Immunisation.

Based on our experience (33) that a reduction in dose of prophylactic from 25 to 5 units resulted in fewer post-vaccinal reactions in a non-Moloney tested group, we chose doses of 5 units for ++ reactors and 1 unit for +++ reactors, + and + reactors receiving a full dose, according to the following plan: — see page 583.

Our object in giving the whole ++ group the first injection without regard to AT_0 was to obtain, without the risk of too much inconvenience to the vaccinated, information as to whether post-vaccinal reactions occurred mainly in the naturally-immune among Moloney-positive persons.

The +, + and ++ groups received the first injection on the same day as the Moloney test was read, while the +++ group, where only persons with less than 0.01 AU previous to immunisation were vaccinated, were given the first injection a week later, as a preliminary titration had to be carried out.

The prophylactic used was that generally used in Denmark for adults, i. e. purified toxoid adsorbed on to aluminum hydroxide. The toxoid was purified by ultrafiltration (32), and contained 620 Lf, per mg N. After dilution in saline mixed with 5 % phosphate buffer solution pH 7.4 and 0.1 % Merthiolate, a sterile suspension of aluminum hydroxide with known Al_2O_3 content was added, so that the prophylactic contained 25 Lf. and 1 mg aluminum per ml. An antigenic test on guinea-pigs, after the Prigge method (30), showed 120–300 protection units per ml. of a dilution containing 50 Lf. and 1 mg Al. per ml.

The injections were given subcutaneously in the supraspinatus region.

Results of Immunisation in ++ and +++ groups.

In order to control immunisation with reduced doses, blood-tests were taken from ++ and +++ reactors two weeks after the third injection and one year later (before the final injection). The results of titration are shown as the geometric mean in table 5. Only persons without measurable antitoxin titre before the first injection are included in the table.

Table 5.
Titre of antitoxin after immunisation.

Moloney results	Units per ml geom. mean					
	Before 1st injection		2 weeks after 3rd injection		1 year after 3rd injection	
	No.		No.		No.	
++	16	< 0.001	16	1.09 (0.09–56.0)*	13	0.10 (0.02–20.0)
+++	34	< 0.001	34	1.02 (0.02–50.0)	29	0.09 (0.01–20)

*) The figures in brackets give the lowest and highest titres in the group.

As will be seen, no titres less than 0.02 units per ml were found in either group two weeks after the third injection. One year later, before the final injection, the mean titres had decreased about ten times in both groups. However, all persons could still be considered protected, since no titres below 0.01 units per ml were found. The mean titres were alike in the two groups, although the +++ group received five times less antigen than the ++ group. This cannot be taken as proof that +++ reactors are easier to immunise than ++ reactors, since the doses-response curve is not known for the two groups.

It has been stated that Moloney-positive persons can be immunised more easily than Moloney-negative persons (inter al. Moloney et al. (24); Burke (6); Fraser (8); Bunch et al. (5)). Having no antitoxin titration from the Moloney-negative group, we undertook a comparison with a non-Moloney tested group immunised two to three months earlier with the same dose of prophylactic as used for the ++ reactors in the present investigation. The means of this group are summarised in table 6.

Table 6.
Antitoxin after immunisation of a non-Moloney tested group.

Units per ml geom. mean					
Before 1st injection		2 weeks after 3rd injection		1 year after 3rd injection	
No.		No.		No.	
30	< 0.001	30	0.33	18	0.13

Statistical evaluation shows that the differences between the means for this group and for the ++ and +++ groups two weeks after the third injection (table 5) are significant ($P = 5\%$ and 3% , respectively). Thus we can confirm the observations of the authors cited above.

A non-Moloney tested group of 17 persons containing just measurable antitoxin titre before immunisation (geometric mean 0.0035 units) and vaccinated in the same way as the ++ reactors, showed more than ten times higher antitoxin production (geometric mean 12.9 units) than the ++ and +++ reactors. Thus, as regards ability to produce antitoxin, these latter would seem to be above those without natural immunity and of unknown Moloney sensitivity, but considerably below persons with even traces of natural antitoxin.

With regard to antitoxin content one year later, there are only slight differences in the means shown in tables 5 and 6, the decrease being greater for the Moloney-positive groups than for the non-Moloney tested groups. We consider that this has no bearing on the Moloney state, but is due to the relation of loss of antitoxin to original antitoxin level (33).

Since the titres before the final injection were all 0.01 units or more, we found it unnecessary to control the results of this injection in extenso, but confined the blood-tests to five persons in the \div group and ten persons in the $+++$ who contained less than 0.05 units before the final injection. All of them showed titres of more than 0.2 units two weeks after the final injection.

Reactions after vaccination.

At the time of the second injection, each vaccinated person was questioned regarding the course of the first injection, and this was also done at the succeeding injections. The complications are classified in the following categories:—

- 0 — none or negligible local and general reactions, e. g. insignificant tenderness and infiltration, or rise in temperature to not more than 38.5° C for less than one day, without other symptoms.
- 1 — moderate local and slight general reactions, e. g. rise in temperature over 38.5° C for not more than one day, without confinement to bed or disability for work.
- 2 — pronounced local tenderness and infiltration and/or general symptoms, such as confinement to bed or disability for work.

Table 7 shows the frequency of reactions after the first injection.

Table 7.
Frequency of post-vaccinal reactions.

Moloney results	Dose of prophylactic	Totals	Degree of reaction		
			0	1	2
\div	25 units	380	354 = 93.2 %	4 = 1.1 %	22 = 5.8 %
+	25 units	77	64 = 83.1 %	4 = 5.2 %	9 = 11.7 % ^{*)}
++	5 units	107	84 = 78.5 %	5 = 4.7 %	18 = 16.8 %
+++	1 unit	59	41 = 69.5 %	4 = 6.8 %	14 = 23.7 %

^{*)} Two of these showed delayed Moloney reaction.

Negative reaction to the Moloney test is not tantamount to non-occurrence of excessive post-vaccinal reactions, there being 5.8 % type 2 reactions in this group.

In spite of the reduced doses of prophylactic, increasing reaction frequency can be seen with increasing Moloney-positivity.

Statistical evaluation of the difference in reactions between the \div group and the three other groups shows significance between the \div group and the ++ and +++ groups, but not between the \div and + groups ($P = 10 - < 0.1$ and < 0.1 % respectively).

We have tried in the following to evaluate these differences further, taking into account the available factors which might influence fre-

quency of reaction, i. e. AT_0 , history of diphtheria, age and sex. As in practice it is type 2 reactions which are of the greatest importance, only these reactions are included in the following analyses.

Influence of AT_0 on frequency of reactions.

Previous investigations (26—29—10—33) have shown marked dependence of AT_0 on frequency of reactions, most reactions occurring in the groups with natural antitoxin. We have dealt in detail with

Table 8.

Influence of AT_0 on reactions after immunisation in the ++ group.

	Total		Reaction 2			
	♀	♂	♀	%	♂	%
< 0.001	5	11	1	20	1	9
0.001—0.1	3	9	1	33	1	11
0.01—0.1	14	24	3	21	2	8
0.1—1	15	21	4	26	4	19
> 1	8	3	0	0	1	33
	39	68	9		9	

this in an earlier work (33) and mentioned some possible explanations. These investigations, however, have only included non-Moloney tested persons. In the present material, only one group — the ++ reactors — is suitable for further elucidation of the problem, as AT_0 here is known and the first injection (5 units) given regardless of AT_0 .

Table 9.

Influence of age, sex and history of diphtheria on reactions after immunisation.

Moloney results	Age	+ previous diphth.						÷ previous diphth.					
		Totals		Reaction 2				Totals		Reaction 2			
		♀	♂	♀	%	♂	%	♀	♂	♀	%	♂	%
+	< 50	7	6	1	14	0	0	76	170	10	13	8	4
	> 50	2	6	0	0	0	0	33	80	2	6	1	1
+	< 50	2	1	0	0	0	0	14	34	3	21	4	11
	> 50	2	0	0	0	0	0	8	16	1	12	1	6
++	< 50	3	3	1	33	1	33	17	42	3	17	4	9
	> 50	5	1	1	20	1	100	14	22	4	28	3	13
+++	< 50	1	5	0	0	2	40	8	31	4	50	5	16
	> 50	0	0	0	0	0	0	6	8	3	50	0	0

Table 8 gives the incidence of reactions within the different AT_0 titres and shows that there are no significant differences or systematic features either for men or women. Comparison of reaction percentages among persons with no measurable antitoxin titre (less than 0.001 units) and the others gives 20 % as against 25 % and 9 % as against 14 % for women and men respectively. In both instances, the differences must be regarded as purely accidental. Therefore, natural antitoxin does not seem to have any bearing on the occurrence of reactions if hypersensitivity to diphtheria prophylactic previously exists.

In Table 9 type 2 reactions are distributed according to previous diphtheria, age and sex.

History of Diphtheria. Comparison of the left and right sections of the table does not indicate any connection between previous diphtheria and frequency of reactions. Claus Jensen (15) and Wohlfeil (38) observed, without taking any other factors into account, that most reactions occurred in children who had previously had diphtheria, whereas Scheibel et al. (33) could only demonstrate this correlation in one group, viz. the group with less than 0.01 units of antitoxin prior to immunisation. The immunised +++ reactors in the present investigation represent such a group, but again reactions are no more frequent among persons with previous diphtheria. However, the different groups are so small that we can only state that history of diphtheria need not be taken into consideration in the following.

Reaction and Age. It has been assumed that age may be a factor influencing frequency of reactions (18—11—26—31), and that most reactions occur in older age-groups. We failed to find any increase in the frequency of reactions in the age-group 15—50 years among non-Moloney tested adults, whereas there was a significant decrease from this age-group to the next (> 50 years), taking into account AT_0 and history of diphtheria (33). On hasty consideration of the ÷ and + groups in table 9, the temptation might be to believe that the same applies to this material. However, statistical evaluation of the difference in the ÷ group, i. e. the group showing the biggest differences and comprising the largest number of observations, gave P as 34 % for women and 28 % for men. Thus the deviations observed are far from being significant. This discrepancy from our previous experience is probably because of the Moloney classification, since the effect of

Table 10.

Influence of sex on type 2 reactions in the different Moloney groups.

Mo'oney reactions	Total		Reaction 2			
	♀	♂	♀	%	♂	%
÷	118	262	13	11.0	9	3.4
+	26	51	4	15.4	5	9.8
++	39	68	9	23.1	9	13.2
+++	15	44	7	46.7	7	15.9

- 3) that the reduction in dosage used here has not been sufficient to prevent a greatly increased reaction frequency in the stronger Moloney-positive groups even though only those persons among the +++ reactors were vaccinated who, judged by their AT_{50} , could be regarded as unprotected or inadequately protected.

Frequency of reactions in Moloney-tested groups compared with frequency in non-Moloney tested groups. It would be of great interest to compare post-vaccinal reaction frequency as a whole in Moloney tested and in non-Moloney tested groups. We have a possibility of carrying out such a comparison, using the previously-mentioned results from vaccination of a non-Moloney tested group (33). Vaccination of the two groups took place within 2—3 months and all were natives of Copenhagen. The persons in the Moloney tested group for the most part*) worked for the same firm — Carlsberg Breweries — and were situated in various places in Copenhagen. In the second group, 27 % worked at the same place (Copenhagen's Town Hall), while the rest were from the same housing complex and had employment either in different parts of the city or in the home.

As mentioned, AT_0 was not measured for all in the Moloney tested group, and therefore we cannot directly examine whether there was any difference in the state of immunity of the two groups before immunisation. However, since the epidemiological conditions must be considered as identical for the two groups, there can hardly be reason to believe that they differed to any degree in this regard. In support of this, it can be mentioned that the two non-Moloney tested groups, which differed with regard to working conditions, showed no difference in natural antitoxin titre (33). A slight tendency in the direction of more naturally-immune in the Moloney tested group is perhaps conceivable, since that group contained mostly persons from the older age groups where natural immunity is most extensive (13—33).

There were an equal number of cases of previous diphtheria (9.5 %

Table 11.

Incidence of sex and age in non-Moloney tested and Moloney tested groups.

Age	non-Moloney tested group				Moloney tested group			
	♀	%	♂	%	♀	%	♂	%
15—29	41	12.0	44	12.9	5	1.9	27	5.7
30—49	254	74.5	235	68.9	166	63.1	388	67.8
50—74	46	13.5	62	18.2	92	35.0	157	27.4
	341	50	341	50	263	31.5	572	68.5

*) Some of the men were employed as drivers and worked part of the time outside the firm's premises.

and 8.5 %). On the other hand, age and sex distribution were somewhat different, as can be seen from the following table: —

The youngest age-groups are, as mentioned, sparsely represented in the Moloney tested group, while, on the other hand, there are more persons in the Moloney tested group than in the non-Moloney tested group in the older age-groups. The figures in the lowest line of the table show that there were relatively most women in the non-Moloney tested group. Statistical evaluation showed that the differences, as far as age and sex are concerned, were significant.

As age has been proved to be relevant to frequency of reactions in the non-Moloney tested group (33), and as sex seems to be of significance to the occurrence of reactions in the Moloney tested group, direct comparison is difficult. It might be maintained that the high number of reactions in the Moloney tested group is due to the peculiar preponderance of women who had strong reactions, but, on the other hand, there are relatively fewer women in the Moloney tested group. The difference in age distribution must be considered as giving fewer possibilities of reactions in the Moloney tested group, since the age-group over 50 years, which in the non-Moloney tested group showed the fewest reactions, is more numerously represented among the non-Moloney tested.

However, taking all this into consideration, some conclusions can be drawn from the following table: —

Table 12.
Frequency of reactions in non-Moloney and Moloney tested groups.

	Number of units in one dose of prophylactic	Total	Reaction	
			No.	%
Non-Moloney tested	12.5—25 Lf.	314	47	15.0
Non-Moloney tested	5 Lf.	368	36	9.8
Moloney tested	varying, see plan, page 11.	835*)	63	7.5

*) +++ reactors who were not vaccinated because they were naturally immune have been included, since we considered it more appropriate in this comparison to estimate reaction percentages on the basis of all persons dealt with in the Moloney investigations.

There are considerably more type 2 reactions among those in the non-Moloney tested group who were vaccinated with 12.5—25 Lf. than in the Moloney tested group ($P < 0.1\%$), but not in those in the non-Moloney tested group who were vaccinated with 5 Lf. (P approx. 20 %). The same result is obtained by comparing only the age-groups 30—35 years which contain the same number of men and women and which, therefore, are directly comparable. In this age-group, among

non-Moloney persons there were $40/217 = 18.4\%$ type 2 reactions after vaccination with 12.5—25 units, and $30/272 = 11.0\%$ after vaccination with 5 units, as against $43/554 = 7.8\%$ in the Moloney tested group. 18.4% and 7.8% are significantly different, but not 11% and 7.8% (P 10—20%). Segregation of naturally-immune +++ reactors and use of graduated doses have, therefore, not reduced the possibility of reaction any more than the use of a dose of 5 units administered to all without previous classification. We have demonstrated previously (33) that this dose causes just as effective an immunity as 25 units, provided that four injections are given instead of three (recall dose included).

Discussion.

The Moloney test, the consequent classification into groups to be vaccinated with different doses, determination of the state of immunity of the strongly Moloney-positive persons (either by Schick test or preferably by AT_0 measurement), the increased number of necessary injections when using smaller doses, and the final examination to ensure that protection has been obtained, are serious burdens in routine-vaccination. Such a procedure, therefore, is only advisable if it offers real advantages to the methods hitherto employed: the same dose, which is known to ensure immunity, for all.

The advantage of using the Moloney test previous to immunisation is closely associated with the question of what should be done with the strongly Moloney-positive reactors.

It has been suggested — and the suggestion carried out — that these persons should be omitted from the vaccination (inter al. 6—19—20), but on account of the considerable number of ++ and +++ Moloney-positive persons who do not contain measurable natural anti-toxin titre, we do not consider this advisable.

Fraser & Moloney et al. (8—24) have carried out reaction-free vaccination of small groups of +++ Moloney-positive persons with doses from 0.01—0.1 ml. O'Brien & Parish (25) have used Toxoid-Antitoxin floccules. Hayman (11) suggests 0.1 ml undiluted toxoid for ++ reactors and a smaller dose or no vaccination at all for +++ reactors. Keller & Harris (16) have immunised Moloney-positive medical students and nurses with diluted vaccine in doses of 0.1—0.25 ml (dilution grades not mentioned) without inconvenient reactions other than moderate rise in temperature and general feeling of malaise. Bunch et al. (5) vaccinated ++ Moloney-positive students with doses from 0.1—1 ml and observed strong reactions to 0.5 ml, whereas +++ Moloney-positive persons showed strong reaction even to 0.2 ml. Intracutaneous injections of 0.1 ml toxoid in dilution 1:100, which led to Schick-negativity after a certain number of injections, could be given without post-vaccinal reactions.

In spite of the use of doses of practically the same size as proposed

by the Canadians (i. e. from 1/25—1/5 of the usual dose), we have experienced a very high percentage of post-vaccinal reactions in +++ reactors. This discrepancy might be due to differences in the prophylactics used (the number of units, for instance, has often not been mentioned), but it is more probable that it is due to age differences, since the Canadians have mostly vaccinated children and young people. Examinations which have included adults (inter al. 16—5) seem to be more consistent with our experiences.

It is obvious to us that satisfactory antitoxin production can be obtained in ++ and +++ reactors by smaller doses than we have used, but the establishment of a dose which, without causing reactions, can ensure immunity is still outstanding and needs great and comprehensive study. In the works quoted, immunisation results are controlled either by the Schick test or by antitoxin titration. Attention is drawn to the not absolute reliability of the Schick test as an individual test, as well as to the difficulty of carrying out routine antitoxin measurement on a large scale. As long as it is necessary to control immunity, our opinion is that the advantage of the Moloney test for use in routine-vaccination is doubtful, particularly because even an absolutely definite negative Moloney reaction does not necessarily exclude the occurrence of severe reactions (approx. 6 % in the present material).

On the other hand, however, the test is a valuable asset in individual cases where it is particularly necessary to try to avoid reactions.

The ideal immunisation method for use in routine vaccination should 1) cause sufficient antitoxin production in all persons so that consequent control of the result is unnecessary, 2) be carried out with a practicable number of injections, and 3) cause a minimum number of severe post-vaccinal reactions.

It has been generally considered, and has been proved by recent investigations by Lawrence & Pappenheimer (17), that hypersensitivity to diphtheria vaccine is partly caused by the bacteria protein content of the vaccine. There is reason to believe that methods, suitable for mass-production, for freeing the vaccine of bacteria protein will soon be brought to light (2—27—28). This would probably mean that a considerable number of post-vaccinal reactions would disappear. However, according to Lawrence & Pappenheimer (17) hypersensitivity to pure diphtheria antigen occurs in a number of persons, mainly among the naturally-immune, but also among Schick-positives. Vaccination without reaction seems, therefore, to be utopian. It seems possible, however, to bring about further improvement in the antigenic effect of the precipitated diphtheria vaccine by quantitative alterations in the mineral carrier, so that the doses generally could be reduced without risk of lessening the effectivity (12—2—3—34). Efforts in these directions would seem to us a better way of finding an ideal mass-vaccination procedure.

Summary.

Sensitivity towards diphtheria prophylactic has been examined in 835 adults in the ages 20—74 years by means of Moloney test. No rise in Moloney-positivity has been observed within these ages, but, on the contrary, a decline in the older age-groups.

Natural antitoxin occurs more frequently in ++ and +++ reactors than in non-Moloney tested groups.

Moloney-positive reactions are more often found among persons with history of diphtheria than among those with no previous diagnosed diphtheria.

÷ and + reactors have been immunised with the full dose (25 units), ++ reactors with 1/5 dose (5 units) and +++ reactors who contained less than 0.01 units of natural antitoxin with 1/25 dose (1 unit), whereas +++ reactors containing more than 0.01 units have not been immunised. The course of immunisation in ++ and +++ reactors has been controlled by antitoxin measurement. Vaccination resulted in higher antitoxin production in these groups than in a corresponding non-Moloney tested group immunised with 5 units.

Obvious connection, but not complete conformity, has been found between Moloney reactions and post-vaccinal reactions, approx. 6% strong reactions occurring among Moloney-negatives.

Increased post-vaccinal reactions have been demonstrated in ++ and +++ reactors, in spite of reduced doses.

Age, previous diphtheria and natural immunity do not seem to be relevant to the occurrence of post-vaccinal reactions, whereas sex appears to be of significance, more reactions having been observed in women than in men. Reaction does not occur more frequently after the second than after the first injection. Reaction after the first injection is not tantamount to reaction after the second.

Post-vaccinal reactions are less frequent in a group classified by and immunised according to Moloney reactions, than in a non-Moloney tested group immunised with 25 units, but are equal to the number of reactions in a non-Moloney tested group immunised with 5 units.

The advantage of the Moloney test in routine vaccination is discussed on this basis.

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OBSERVATIONS ON EXPERIMENTAL DENTAL CARIES

V. THE EFFECT OF CERTAIN QUINONES WITH, AND WITHOUT VITAMIN K ACTIVITY

By *Humberto Granados, Johs. Glavind and Henrik Dam.*

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The studies on the inhibitory action of certain quinones with, and without vitamin K activity on acid formation in saliva-glucose mixtures, as well as the experimental and clinical investigations on the effect of vitamin K in the prevention of dental caries, have been recently reviewed by Dam (1948). He has pointed out the contradictory results obtained and conclusions arrived at in these studies, and the need for further experimental investigations on this subject.

Considering this fact we have carried out some studies on the effect of certain quinones with, and without vitamin K activity on experimental dental caries. We are here reporting the results of these investigations.

Experimental.

Ninety-eight syrian hamsters, between 21 and 24 days of age, from litters of a colony maintained on Purina Laboratory Chow¹ and raw milk, were litter-mate distributed into seven groups of 14 animals each (8 males and 6 females). The animals were reared in screen bottom cages without bedding for 140 days on the following basal diet to which was added in each case the quinone indicated in table 1: ground yellow corn² 25 %, sucrose 25 %, corn starch 20 %, powdered whole milk 24 %, ether-extracted yeast 5 %, and salt mixture³ 1 %. In table 1 can be seen that 6 quinones were used, the first 3 with⁴, and the last 3 without vitamin K activity; all these quinones

were supplied in equal molecular concentrations. The groups were given water ad libitum, and were weighed weekly. These experiments were carried out between the months of July and November.

On completion of the experimental period the prothrombin times of blood taken from the external jugular vein (two determinations for each animal) were determined in all the groups by the technique of Larsen and Plum (1941). The animals were then sacrificed and autopsy was performed on them. After fixation in 10 % formalin the jaws were prepared for examination in the usual way (Granados, Glavind and Dam, 1948). The earious lesions were recorded and scored using the chart described by Keyes (1944).

Results.

The animals from all the groups exhibited the same health appearance; the growth rate, however, was not the same in all of them. Fig. 1 shows that group 1 (control without any quinone), 3 (calcium-synkavit), and 5 (2,3-dichloro-1,4-naphthoquinone) exhibited essentially the same growth rate. Group 2 (sodium-synkavit) exhibited slightly higher growth than groups 1, 3 and 5. The opposite was true in group 4 (menadione), which showed a slightly lower growth, and in groups 6 (hydroquinone) and 7 (benzoquinone), which exhibited growth rates lower than those of the other groups. These last quinones, therefore, appeared to be slightly toxic at the levels given.

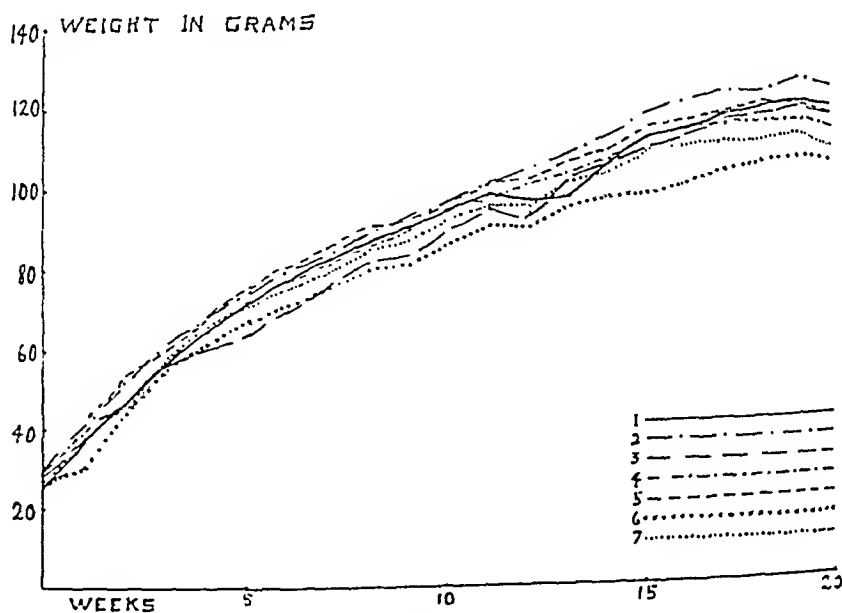


Fig. 1.
Average growth curves of the seven groups.

In table 1 the average prothrombin times of the seven groups are given. It is clear that no significant differences occurred among any of them. It may be noticed that 2,3-dichloro-1,4-naphthoquinone²,

Table 1.

Kind of quinone given to and prothrombin times of each of the seven groups.

Group No.	Kind and quantity of quinone added in mg per 100 g of food	Average prothrombin times in seconds
1	None	23.4
2	104 mg tetrasodium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (sodium-Synkavit)	23.7
3	70 mg dicalcium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (calcium-Synkavit)	22.6
4	28 mg 2-methyl-1,4-naphthoquinone (menadione)	23.1
5	37 mg 2,3-dichloro-1,4-naphthoquinone	22.4
6	18 mg hydroquinone	23.6
7	18 mg benzoquinone	22.8

the high toxicity of which toward yeasts can be reversed competitively by vitamin K over a limited range of concentration (Woolley, 1945), did not produce hypoprothrombinemia. On the other hand, none of the three quinones with vitamin K activity (groups 2, 3 and 4) shortened the prothrombin times. This shows that either the 25 % dietary corn and/or the intestinal flora supplied enough vitamin K to keep the prothrombin time of the adult hamsters normal so that no further decrease could be obtained by adding relatively high supplements of vitamin K to the diet.

The autopsy of the various groups revealed no gross changes other than those found in the oral cavity. Table 2 shows the caries activity in the various groups. Beneath the average number of carious molars, carious lesions, and caries score are presented the standard deviations of the means of the males, females, and males and females together. It is apparent that the caries extent (average caries score) was nearly the same in all the groups. On the other hand, as compared with group 1 (control) the caries incidence (average number of carious lesions) was slightly lower in groups 2 (sodium-Synkavit) and 4 (menadione). Groups 3 (calcium-Synkavit), 5 (2,3-dichloro-1,4-naphthoquinone), 6 (hydroquinone) and 7 (benzoquinone) exhibited nearly the same caries incidence as group 1.

In the present studies the differences in caries incidence between the control group and those which received either sodium-Synkavit or menadione are of no statistical significance.

Table 2.
Caries activity in the seven groups.

	Group 1			Group 2			Group 3			Group 4		
	7*	6	13	8	6	14	8	6	14	8	5**	13
Number of experimental animals			100			100			100			100
Percentage of animals affected												
Average number of carious molars.	7.7	9.5	8.5	7.1	9.1	8.0	8.6	8.3	8.5	6.5	7.6	6.9
Standard deviations	± 1.00	0.88	0.69	0.89	0.83	0.68	1.27	1.23	0.86	0.84	0.81	0.60
Average number of carious lesions.	10	12.7	11.2	8.1	10.0	8.9	10.8	10.0	10.4	7.5	9.2	8.2
Standard deviations	± 1.64	2.05	1.28	1.13	0.96	0.78	1.41	1.29	0.82	1.12	0.85	0.77
Average caries score.	6.9	8.1	7.5	5.8	6.9	6.3	7.8	7.0	7.5	5.1	6.7	5.7
Standard deviations	± 1.09	1.39	0.85	1.19	1.00	0.81	1.29	1.16	0.87	1.11	0.64	0.70

	Group 5			Group 6			Group 7		
	8	6	14	8	6	14	8	6	14
Number of experimental animals			100			100			100
Percentage of animals affected									
Average number of carious molars.	8.1	10.2	9.0	9.3	9.0	9.1	8.3	8.5	8.4
Standard deviations	± 0.52	0.91	0.55	0.79	0.93	0.61	0.86	0.92	0.61
Average number of carious lesions.	9.6	15.3	12.1	11.9	11.9	11.9	10.0	9.3	9.7
Standard deviations	± 0.80	1.20	1.07	1.62	1.27	1.04	1.25	0.92	0.79
Average caries score.	5.9	9.8	7.6	7.5	7.7	7.6	6.4	6.1	6.4
Standard deviations	± 0.70	0.67	0.71	1.25	1.12	0.83	1.10	1.34	0.84

*) One of the 8 original males of this group died in the course of the experiment.

**) One of the 6 original females of this group died in the course of the experiment.

Table 2 also shows that, as a general rule, the standard deviations are smaller when calculated in a whole experimental group, irrespective of sex, than when calculated in the corresponding subgroups of males and females separately. This suggests that in similar experiments on caries in hamsters in which animals of both sexes are used in each group, comparison between the various groups should be made irrespective of sex. Likewise it shows that in the present studies there was no difference between the sexes with respect to caries susceptibility.

Discussion.

Hatton et al. (1945) studied the action of several levels of menadione and of its bisulfite addition product on experimental dental caries in rats, for 3 generations. They found no differences in the incidence of caries between the test and control groups. Likewise, we have found that in hamsters none of the various quinones tested decreased caries activity to any significant extent.

Fosdick (1948) has recently discussed the theoretical and practical considerations which led him to suggest the use of menadione in the prevention of dental caries. He showed (1942) that 2-methyl-1,4-naphthoquinone is able to inhibit acid production in glucose-saliva mixtures. Later Armstrong et al. (1943) demonstrated that this effect of menadione is due to the quinone structure of the compound, and showed that the same results could be obtained with other quinones possessing little or no vitamin K activity.

Under the conditions of these experiments the differences in caries activity among the various groups were insignificant. Thus none of the quinones tested decreased caries activity to any significant extent. It might prove worthy to test again the effect on caries of various quinones with and without vitamin K activity, and with markedly different ability to inhibit acid production *in vitro*, using larger amounts of the compounds than those used in the present studies. Although in case of obtaining favorable results the implications of such an experiment would not favor the use of quinones, due to their toxicity, in the prevention of dental caries, a study of this kind could show how far the ability of certain quinones to inhibit acid production *in vitro* is paralleled by a corresponding ability to decrease caries incidence. This might help in the understanding of any fundamental relationship between acid action and the carious process itself.

Summary.

The effect of 3 quinones with (sodium-synkavit, calcium-synkavit, and menadione), and 3 without vitamin K activity (2,3-dichloro-1,4-

naphthoquinone, hydroquinone, and benzoquinone) on dental caries activity has been studied in hamsters, with a litter-mate control group. Under the conditions of the experiments none of the groups which received quinones exhibited any significant decrease of caries activity as compared with the control group. Some implications of these studies are discussed.

1. From Ralston Purina Company, St. Louis, Mo., U. S. A.
2. 100 % of the ground yellow corn passed through a 60-mesh screen.
3. The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 556 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ per 100 g.
4. We thank F. Hoffmann-la Roche & Co., Basle, Switzerland, for the kind supply of the sodium-Synkavit, calcium-Synkavit and menadione used in these experiments.
5. We thank Naugatuck Chemical, Division of United States Rubber Company, Naugatuck, Conn., U. S. A., for the kind supply of the 2,3-dichloro-1,4-naphthoquinone used in these studies.

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CERVICAL LYMPH NODE TUBERCULOSIS AND THE TONSILS

By *Mikko Pentti*.

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The question of the patogenesis of tuberculosis of the cervical lymph nodes, especially in relation to tonsillar tuberculosis, has been treated by many investigators. It is closely associated with the question of primary tonsillar tuberculosis in general, its frequency, and significance as portal of entry for tuberculous infection. There have been very differing opinions on the matter, extreme views having been put forward, but so far no agreement has been reached.

First after *Ranke* had accurately defined the primary complex, the studies have been brought to a solid foundation. Through their careful autoptic investigations *Ghon* and *Winternitz* showed that it was possible to establish the primary complex in almost every case, and that its site, in by far the greatest number of cases, was in the lungs. Tuberculosis of the tonsils, which was comparatively frequent, was in most cases found to be secondary, haematogenous. Some unmistakable primary cases of tonsillar tuberculosis were established as well, but their number was small.

Schürmann's and *Kleinschmidt's* remarkably thorough investigations of the victims of the notorious Lübeck catastrophe give us a detailed picture of primary tuberculosis and its pathology. In these cases the primary affection was fairly often situated in the pharyngeal ring, naturally because of the oral manner of the infection. The investigations showed that the primary affection in the tonsil forms an ulceration, which, however, in about 50 per cent of the cases was invisible to the eye. They found that the ulceration tended to heal fairly rapidly, and afterwards the scar was either seen only with difficulty or invisible. It also appeared that certain groups of lymph nodules corresponded to definite parts of the mouth cavity, and that the tuberculous process spread quickly from one group of nodules to another and also to the opposite side of the throat. When the primary affection had its site in the tonsil, which occurred quite often, the

upper deep cervical lymph nodes were first affected in the trigonum caroticum. In all cases the lymphomas were massive and tended to caseate rapidly and to suppurate.

It is easy to understand that in the case of extrapulmonary types of tuberculosis especial attention has been devoted to the pharyngeal ring. *Otto* studied histologically 45 pairs of tonsils, mainly extirpated from children, in cases where tuberculosis had been established clinically. He found tonsillar tuberculosis in 74 per cent of the cases, which, however, was primary in only one case. All the rest he considered haematogenous. It should be mentioned that *Otto* also examined in 23 cases the bone marrow in the base of the skull histologically, finding there tubercles in 16 cases. This obviously speaks strongly in favour of a haematogenous aetiology of tonsillar tuberculosis. *Schlittler* also examined extirpated tonsils in cases where tuberculosis of the cervical lymph nodes reasonable was strongly suspected, and established tonsillar tuberculosis which he considered primary in 48 cases out of 98, i. e. about 50 per cent. Yet other investigators do not agree with him about the primary origin of the disease but declare that in his cases it more likely was secondary, haematogenous.

In the Scandinavian countries, in recent years, many investigators have declared that primary tonsillar tuberculosis is fairly frequent and have emphasized its importance as a gate of infection in the pathogenesis of extrapulmonary tuberculosis in general, and especially in the genesis of cervical lymph node tuberculosis. *Ellonen* published in 1943 a study of 47 cases of bone and joint tuberculosis in children. In the histological examination of the extirpated tonsils he demonstrated tonsillar tuberculosis in 25 per cent of the cases and set it down as primary. It should be noted, however, that in these cases no X-ray examination was made of the lungs. Nor did the clinical picture show the characteristics of primary tuberculosis, for the gland component, which unquestionably forms a part of the primary complex, was negligible and was even in some cases missing. The histological picture also differs from the primary tonsillar tuberculosis described by *Schürmann* and *Otto*, not to speak of the fact that the primary affection may hardly be supposed to remain unchanged in the tonsil for years, which would have been the case in these instances.

Arvid Wallgren declares that in Sweden, where according to some investigators, about 20—30 per cent of the tuberculous lymph cervical nodes are due to bovine infection, they belong to the primary complex. In support of his statement he refers to the observation made in Sweden that the symptomatic occurrence of erythema nodosum is often encountered in connection with lymph node tuberculosis. It should be mentioned that erythema nodosum in connection with this affection is unknown in Finland. It is open to question whether this is due to the fact that bovine tuberculosis is practically non-existent in this country.

Vuori published in 1945 a research on 50 cases of tuberculosis of the cervical lymph nodes, in which tonsillectomy was carried out and the tonsils were examined histologically. He found tonsillar tuberculosis in 7 cases, in 5 of which it was bilateral. *Vuori* considers, though with some reservations, the tuberculosis as primary, but his opinion does not seem sufficiently well-grounded. Many facts speak for secondary, haematogenous tuberculosis.

In 1948 *Beck Mathiesen* published an investigation comprising 122 cases, mostly children, the extirpated tonsils of whom had been found tuberculous. In view of the histological picture he supposes that in 90 per cent of cases it is a matter of primary tonsillar tuberculosis. The motivation of his conception is, however, so slightly assuring that even his conclusions are subject to great doubt.

In Finland many observations seem to indicate that cervical lymph node tuberculosis has become more and more rare, and it seems that its incidence has decreased more rapidly than that of tuberculosis in general. It has not yet been discovered to what this phenomenon is due, but it seems fairly sure that the improvement of general hygiene has some share in it. One cannot, therefore, help being struck by the marked frequency of cervical lymph node tuberculosis during the recent 1941—44 war in this country. In the autumn of 1942 an ever increasing number of these cases turned up at the military hospital where I was working. This fact gave me the opportunity to use the material for detailed research in order to throw additional light on the question of the pathogenesis of tuberculosis of the cervical lymph nodes, especially in its relation to the tonsils. I made a histological and bacteriological study of 30 pairs of tonsils extirpated from soldiers in cases where cervical lymph node tuberculosis had been established clinically. Serial sectioning was made of one half of each tonsil, and they were examined by microscope, the other half I examined for the presence of tubercle bacilli by grinding it and treating the mass with sulphuric acid, after which it was neutralized, and about 1 ml. of the emulsion was injected intraperitoneally into guinea-pigs, which were dissected after 6 weeks. The clinical study of the cases was made as thorough as possible, tuberculin tests were made and the lungs were examined by means of roentgenograms with the exception of two cases where only X-ray screening was carried out. All the examined patients were tuberculin-positive.

The age of the patients varied between 19 and 33 years. Tuberculosis occurred in the family history of 16, i. e., in about 50 per cent of the cases. 9 of the patients had previously had tuberculosis, 8 of them lymph node tuberculosis and 1 tuberculosis of the joints. X-ray of the lungs showed definite evidence of tuberculosis in 24 cases. In most of these cases calcification was noted, remnants of pleurisy or cicatricial changes of the parenchyma in some cases. There were no findings pointing to active pulmonary tuberculosis. The lung findings

were thus positive in 80 per cent of the cases. It should be noted, however, that a negative lung finding does not exclude the possibility of tuberculosis, since, as we well know, a primary affection in the lungs, that has been cured, in many cases cannot be established roentgenologically.

Tonsillar tuberculosis was disclosed in one case only.

The patient was a 20-year-old soldier whose family history was negative as to tuberculosis. He was in the hospital from 10/7 to 18/12/42 with the diagnosis: tuberculosis of cervical lymph nodes and tonsils. Earlier his general health had been good. He was summoned for military service on 20/9/41. In the early spring of 1942 he complained of tiredness. In June the lymph nodes in the neck began to swell. On admission to the hospital his general condition was good, S. R. 19, Pirquet +. On the right side of the neck he had a chain of lymph nodes of the size of the tip of a finger and smaller, on the left, nodes of bean-size. X-ray film of the lungs: In the left apex and in the II i. c. patches of shadow, 2 cm. in diameter. The patient was given X-ray treatment on the lymph nodes. During the treatment he developed suppurating otitis, which, however, healed without complications. On 28's bilateral tonsillectomy was performed. The tonsils were of the size of an almond, and adhered to their base. The lymph nodes diminished gradually and the patient was discharged from the hospital on 18/12.

Serial sections were made of a part of the tonsils; another part was treated in the way described above and inoculated into a guinea-pig, which was killed after 6 weeks. On autopsy, masses of tubercles were discovered in the abdominal organs and lungs of the guinea pig. It was not possible to determine the type of the bacilli. Tubercles were found in both tonsils on the histological examination. Professor of pathology Arno Sarén, gave the following description of the histological finding:

»There is an extensive tuberculous process in both tonsils, consisting of solitary and conglomerate tubercles. In the tubercles there are mainly epithelioid cells, but also Langhans giant-cells. There is only sparse necrosis in the centre of tubercles, mainly in the form of fibrinoid necrosis. The tubercles are scattered throughout the lymphatic tissue of the tonsil, but they appear in abundance even deep into the peritonsillar connective tissue. Some of the tubercles are immediately beneath the surface epithelium, which has desquamates in many places and is sometimes missing entirely, in which case the surface is covered by a necrotic layer infiltrated with granulocytes. There is no increase in the fibrillary connective tissue round the tuberculous infiltrates, nor elsewhere in the tonsil.«

The histological findings correspond with the description given by many investigators (*e. g.*, *Otto*) of haematogenous tonsillar tuberculosis. On the other hand, it differs from the picture given by *Schürmann* of the primary tuberculosis in the Lübeck cases, which showed a marked ulcerous formation in the tonsils. In other respects, too, my case presents the characteristics peculiar to primary tonsillar tuberculosis, firstly because the cervical lymph nodes were rather small, no bigger than the tip of a finger, while in primary complexes they are massive, caseating and suppurating early, also, secondly, because changes were noted in one lung which were clearly pointing to tuberculosis.

I think there is no doubt that we have to do with simultaneously haematogenous tuberculosis in the tonsils and the cervical lymph nodes. A coincidence of this kind is by no means rare, as many earlier investigations have shown. Some authors, *e. g.*, *Bandelier* and *Zöllner* suppose this coincidence to be due to the fact that because of the very common occurrence of ordinary infections in the tonsils and their lymph system a so-called locus minoris resistentiae is formed in this system, preparing ground for a tuberculosis infection. The striking frequency of cervical lymph node tuberculosis in Finland among soldiers during the war seems to indicate that the exceptional conditions at the front with overcrowded and primitive accomodation, with necessarily unsatisfactory hygiene and conditions favouring the spread of ordinary infections, constituted the essential cause. The problem of the pathogenesis of lymph node tuberculosis is, however, not definitely solved in this way, as there are evidently other contributory factors. It still remains to be shown what part, for example, the constitutional factors play.

Though my material is small, I feel justified in drawing certain conclusions. The fact that out of 30 cases of cervical lymph node tuberculosis tonsillar tuberculosis was established only in one case, where it evidently was not primary, and that in 80 per cent of the cases there were changes in the lungs which were obviously tuberculous, speaks for the supposition that cervical lymph node tuberculosis in by far the most cases is secondary, haematogenous, at least in a country where bovine infections is very rare. Where bovine infection is common it may be otherwise. In investigations from which the opposite conclusions are drawn and tonsillar tuberculosis has been considered primary, whether it is a question of cervical lymph node tuberculosis or other extrapulmonary tuberculosis, the authors have not always been critical enough in their observations of the characteristics peculiar to a primary complex. Not all the investigators seem to have paid sufficient attention to the fact that before we may speak of a primary affection situated anywhere outside the lungs, we must exclude with the greatest possible certainty the existence of any tuberculous changes in the lungs.

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Summary.

The writer has examined histologically and bacteriologically the extirpated tonsils of 30 soldiers who clinically had been found suffering from cervical lymph node tuberculosis. Only in one case was the tonsillar tuberculosis established, which in all probability was secondary, haematogenous. The writer draws the conclusion that the cervical lymph node tuberculosis, in adults at least, is most frequently secondary, haematogenous, and that primary tonsillar tuberculosis appears rather rarely — quite in contrast to what many investigators in the Scandinavian countries have been inclined to assume.

age in the non-Moloney tested material was only demonstrable in the groups with AT_0 over 0.01 and, as mentioned, these very groups are likely to be weakly represented among Moloney-negatives. In the ++ and +++ groups, the frequency of reactions varies in the different age-groups. Thus there is nothing here either to indicate that older people who have shown hypersensitivity to diphtheria prophylactic have less chance of reacting than younger persons.

Reactions and Sex. In nearly all the groups, there is a greater frequency of reactions in women than in men. This observation has been more closely examined on the basis of Table 10, where the classifications which have proved unnecessary are omitted.

In these large groups, the biggest reaction frequency is again seen among women, but not the same persons reacted. The differences are significant (P less than 5 % for two of the groups). We have not previously found any difference in reaction frequency between the sexes (33), nor have we seen reference made to it in the literature. We cannot give any adequate explanation of this peculiarity: there might be certain connection with the fact already mentioned, that women seem to preserve hypersensitivity to diphtheria antigen as expressed by Moloney reaction longer than men.

Final comments on incidence of reactions in the different Moloney groups. After having seen that, in this material, sex is relevant for post-vaccinal reactions, the frequency of reactions in the different Moloney groups is examined, taking this factor into consideration. Table 10 shows that increase in frequency of reaction with advance in Moloney-positivity can be seen in both sexes. The difference between the ÷ and the ++ and +++ groups is highly significant ($P \leq 1/1000$) but not between the ÷ and the + groups.

Reactions after further injections. In the preceding, we have dealt only with reactions after the first injection. With regard to reactions after further injections, we shall only make brief mention of the relation between reactions after the first and second injections. 18 women had type 2 reaction after the first injection but no reaction after the second, while only on one occasion was type 2 reaction seen after the second injection where there had been no reaction after the first injection.

As far as men are concerned, reactions occurred equally frequently after the first and second injections, but not the same persons reacted. Reactions after the second injection were not found more frequently among women than among men. On the whole, it is our impression that the first injection of prophylactic did not induce hypersensitivity but rather seemed to cause a certain desensitisation.

Briefly, the investigation has shown: —

- 1) that severe reactions could not be avoided in the Moloney-negative group,
- 2) that even a weak skin reaction means increased possibility of post-vaccinal reaction.,

pressure in the tissue may be assumed to differ from the tension in the rest of the parenchyma. *Teir* (1948) states, however, that he has not been able to alter the nuclear class formation in the external orbital gland of the rat by extirpation of the capsule of the gland, nor by attempts to produce shrinking scar tissue by cauterization or extirpation of parenchyma, nor by trying to produce local changes in the pressure by sealing the gland with paraffin. On the other hand, in extensive experiments with X-ray treatment of the gland he succeeded in disturbing the normal nuclear class formation.

Another hypothesis has been advanced by *Jacobj* who thinks that the localization of the different nuclear sizes depends on the different functional states of the parenchymal sections, especially in the liver lobules within their radiate structure. For the large-nuclear cells are found to be most numerous in the intermediate zone of the lobules, where the metabolism is assumed to be most lively. In the pancreas of the rat *Jacobj* (1925) found the higher nuclear classes at the larger blood vessels. Correspondingly, *Birkenmaier* (1934) found large-nuclear cells in highly vascularized parts of the liver in the blackbird (*turdus merula*) and in the more richly vascularized hilus region of the lachrymal gland in mouse. From these observations *Jacobj* (1942) finds it reasonable to conclude that the nuclear class formation depends also on the local vascular supply. This view may be harmonized with a third hypothesis about the eliciting factors in the nuclear class formation advanced by *Teir*: »Es ist aber möglich und sogar wahrscheinlich, wie dies auch aus vielen Experimenten hervorgeht, dass auch viele chemische Stoffe solches Verdoppelungswachstum hervorrufen können (siehe z. B. *Langer* 1942). Wenn dasselbe unter normaler Entwicklung stattfinden würde, könnte man von Hormonen sprechen«.

The last-mentioned hypothesis may be investigated in the following experimental studies. These experiments were carried out in order to see whether the nuclear class formation in the liver is influenced by decreased secretion of anterior pituitary growth hormone, and whether the supply of purified growth hormone to individuals with lowered secretion of pituitary growth hormone may have influence upon the nuclear class formation.

Material and Technique.

The experimental animals here employed belong to a mouse strain (B) with recessive anterior pituitary hypoplasia, thoroughly described by *Francis* (1944). Under expedient breeding this strain yields litters comprising normal mice as well as mice with anterior pituitary hypoplasia. The latter do not attain spontaneously a body weight over 5 to 8 g., presenting thus hereditary pituitary dwarfism. Of this strain, litters have been picked out with an age difference of $\frac{1}{2}$ —2 weeks, from birth to the age of 28 weeks. From each litter, normal mice were picked out for controls together with 2 pituitary dwarf mice, one of which was treated from the age of 4 weeks with a standardized preparation of anterior pituitary growth hormone*) in daily doses of 0.1—0.3 ml. on 6 days of the week. This dosage gives a distinct increase in growth (*Fonss-Bæch*, 1947).

*) The pituitary growth hormone (growth promoting pituitary extract), phyol, was obligingly placed at my disposal by Alfred Benzon, Ltd., Copenhagen.

In order to avoid variation of factors that may have influence on the size of the nuclei, all the mice were raised in the same quarters, at a constant diet, and decapitated at the same time of the day. For determination of the size of the nuclei the specimens (liver) were fixed in 10 % formalin solution and embedded in paraffin. The blocks were cut in sections of $8\ \mu$, which were stained with hematoxylin and eosin. The preparation of the specimens was kept constant throughout.

The size of the nuclei was determined by ocular micrometry: Zeiss' apochromatic 120 (numerical aperture 1.30), Zeiss' binocular tube 2, Zeiss' measuring ocular 7.

In order to obtain a representative selection of mononuclear and binuclear cells for nuclear measurement, only those cells were measured whose center fell inside the area of the ocular measure when the microscopic stage was moved from side to side. Thus, after the cells in a $6\ \mu$ wide band of the section had been measured the stage was moved $100\ \mu$ forwards, and the cells in a new band of section were measured, until 250—500 cells were measured, the number of measured cells being increased when the dispersion of the values for the nuclear size seemed large. The nuclear size is determined after the same principle as employed in similar previous investigations (Jacobs, Teir), the largest nuclear diameter and the smallest diameter perpendicular on the larger one being measured, corresponding to the largest optical nuclear section with the measuring unit of 0.5 measuring interval ($1\text{ M. I.} = 0.916\ \mu$). The average of two measurements of the same nucleus is recorded as signifying the linear extension of the nucleus. The frequency distributions of nuclear diameters — for mononuclear and binuclear cells, respectively — are plotted in double logarithmic coordinate systems with the nuclear diameter groups in M. I. logarithmically along the axis of abscissas, and the observed number of cells with the respective nuclear diameter is plotted logarithmically along the ordinate. Biological arguments for a logarithmic presentation of nuclear diameters are advanced by Hintzsche (1946), and it seems to facilitate the statistical treatment of the nuclear diameters, as these may be looked upon as showing a logarithmically normal distribution. Also logarithmic plotting of the number of cells observed facilitates a statistical analysis of the nuclear classes, which, if distributed normally, appears as parabolas.

Besides, the observations are recorded in Tables 1—2 where the number of cells with the given nuclear diameter is entered in rows for each specimen. Whenever there is a parallel shift of the row, it indicates the division of the observations into nuclear classes. In the overlapping fields of the rows the sum of the two figures gives the number of cells observed.

Results.

The nuclear class formation in the livers of 27 normal controls shows no particular deviations from previous investigations. For mononuclear cells (Table 1) the distribution of nuclear diameters is logarithmically normal at birth. Mice of 3 weeks show a distribution composed of two separate distributions (nuclear classes): K_1 and K_2 . Mice from 0—2 weeks old show skew distribution that may be looked upon as composed of two distributions, of which the one with the high diameter values (K_2) is represented by few observations. From the age of 9 weeks the mice show a distribution composed of three

Table 4.

[illegible]

Table 1 (continued).
Mononuclear liver-cells (normal mice).

Age in weeks	9	10	11	12	13	14	15	16	17	18	20	22	26	28
Specimen No.	2016	1451	1368	1481	1356	1361	1514	1266	1618	1427	1439	1298	1353	1382
M 1 (0.916 ρ)	y = log. M1 × 1000													
4½	653													
4¾	677													
5	689													
5¼	720													
5½	740			1						3				
5¾	760	4	2	3						9				
6	778	5	3	12	1		1		4	11		6		
6¼	796	11	19	6	36	4	4	13	1	38	15	13	4	7
6½	813	11	25	31	37	21	3	49	10	61	20	40	19	12
6¾	829	2	19	89	22	41	25	46	33	42	25	53	9	37
7	845		11	71	3	48	18	36	25	5	8	31	1	12
7¼	860	4	2	21	1	25	45	15	11	2	4	1	7	5
7½	875	6	4	6	7	13	21	2	1	1	4	1	1	
7¾	889	21	5	3	40	5	1	3	3	5	12	16	6	1
8	903	33	16	1	55	1	1	13	9	41	32	12	36	1
8¼	916	10	21	11	52	25	5	25	37	50	41	32	60	7
8½	929	15	35	48	21	50	22	50	70	42	41	49	73	18
8¾	912	9	20	49	4	69	17	42	69	21	28	35	30	20
9	951	11	11	45		30	51	21	30	11	18	21	17	22
9¼	966	4	5	21	3	18	26	16	16	3	10	11	8	19
9½	978	4	1	9	6	9	19	8	5		6	4	2	16
9¾	989	6	2	11	1	3	1	3	3	1	1	6	2	12
10	1000	7	1	26	3	1	3	1	5	8	4	9	1	2
10¼	1011	5	1	22	2	2	6	7	13	9	10	20		1
10½	1021	7	5	15	7	8	21	15	20	19	10	30	3	4
10¾	1031	2	3	8	6	4	4	18	20	17	19	20	31	1
11	1011	2	2	6	6	4	10	10	19	14	23	11	17	2
11¼	1051			5	2	4	14	11	11	6	10	13	8	2
11½	1061		1	6	1	1	26	5	11	2	15	3	4	4
11¾	1070			2	3	19	4	4	1	5	3	2	6	7
12	1079		1	2		9		3		1	2		7	3
12¼	1088					8	1							
12½	1097				1	1				2				
12¾	1106							1					1	1
13	1114				1									1
13¼	1122				3					1		2		
13½	1130					1		1			2	2		
13¾	1138		1			1		1	1			1		
14	1146						2				1	3	2	1
14¼	1151					1				1				
14½	1161					1	2	1			1			
14¾	1169									1				
15	1176						1						1	
15¼	1183						4							
15½	1190						2							
15¾	1197						1							
16	1201									1				
16¼	1211													
16½	1218				1									

nuclear classes (K_1 , K_2 and K_4) and the suggestion of a fourth one (K_5) as shown in Fig. 1. In mice between 4 and 8 weeks old the nuclear class with the highest values for the diameter (K_2) shows vary-

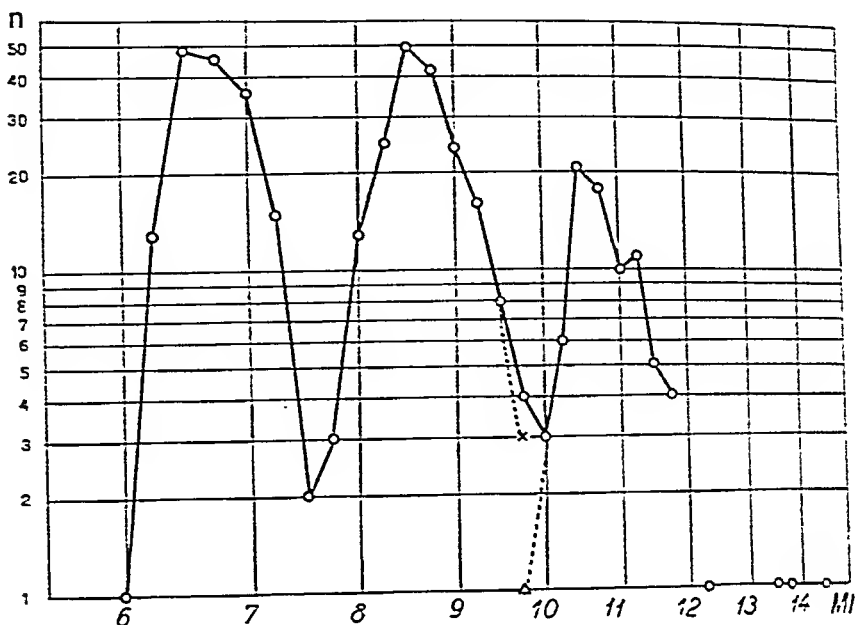


Fig. 1.

Nuclear diameters of normal mouse (1514).

ing degrees of skewness due to a third component (K_4), which is represented only by a few observations.

In the liver of normal mice binuclear cells are far from being as frequent as mononuclear cells (16 mice, 8 weeks old or more, showed in the uncorrected material $16.2 \pm 0.8\%$ polynuclear cells). The binuclear cells are encountered from the age of 2 weeks and are suggestive of a distribution, $2K_1$, from the age of 3 to 4 weeks. From the age of 4 to 5 weeks we meet with a bimodal distribution, comprising two nuclear classes, $2K_1$ and $2K_2$; and from the ninth week it is made up of three nuclear classes: $2K_1$, $2K_2$ and $2K_4$.

In 27 pituitary dwarf mice the liver shows for mononuclear cells — in all the specimens — either one apparently logarithmically normal nuclear diameter distribution, K_1 , or a skew distribution that may be interpreted as composed of two nuclear classes, of which the one with the high diameter values, K_2 , is represented only by few observations (Table 2 and Fig. 2). This picture appears not to be dependent upon the age within the period here investigated: from birth to 28 weeks. Binuclear cells appear also in pituitary dwarf mice from the age of 2 weeks, with a suggestion of distribution from the age of 4 weeks. Only in 3 cases is the presence of a higher nuclear class, $2K_2$.

Table 2.
Mononuclear liver-cells (pituitary dwarf-mice).

Age in weeks	0	1	2	3	4	5	6	7	7	8	8	9	9
Specimen No.	2128	2141	2146	1473	1458	1710	1499	1491	1788	1226	2139	1500	2015
M 1 (0.916 μ)	y = log. M1 × 1000												
4 $\frac{1}{2}$	653												
4 $\frac{3}{4}$	677												
5	699						4			2			1
5 $\frac{1}{4}$	720	4			4		19		3	2	8		11
5 $\frac{1}{2}$	740	12	2	2	12	8	32	3	14	10	13	5	11
5 $\frac{3}{4}$	760	16	9	8	29	27	44	15	34	41	18	37	75
6	778	19	12	36	10	61	13	56	29	69	85	72	72
6 $\frac{1}{4}$	796	29	11	57	24	67	4	92	14	52	15	41	53
6 $\frac{1}{2}$	813	44	23	46	7	32	1	19	1	24	19	18	15
6 $\frac{3}{4}$	829	6	24	29	2	10		15	1	4	7	3	6
7	845	2	9	6		1		2		1		2	2
7 $\frac{1}{4}$	860		3	1	1			1		1	1	1	2
7 $\frac{1}{2}$	875		1	2	2	3				1	1	2	1
7 $\frac{3}{4}$	889		1	3	2				1	1		3	1
8	903			3	3	1		2				1	
8 $\frac{1}{4}$	916			1	3			2					1
8 $\frac{1}{2}$	929					1	1						
8 $\frac{3}{4}$	942												

Age in weeks	10	11	12	13	14	15	16	16	17	18	20	22	26	28
Specimen No.	1453	1470	1480	1355	1366	1543	1516	1265	1647	1426	1438	1297	1352	1384
M 1 (0.916 μ)	y = log. M1 × 1000													
11 $\frac{1}{2}$	653						1							
11 $\frac{3}{4}$	677		1				1							1
12	699	2		1		2							1	7
12 $\frac{1}{4}$	720	8			5	8	6		2		1		2	25
12 $\frac{1}{2}$	740	22	2	1	12	26	16	5	1	15	2	5	3	61
12 $\frac{3}{4}$	760	44	9	6	52	54	51	34	29	32	24	11	40	87
13	778	25	21	36	80	19	73	68	57	70	63	81	94	14
13 $\frac{1}{4}$	796	9	37	70	57	30	59	58	59	56	75	67	63	16
13 $\frac{1}{2}$	813	3	18	67	28	8	45	21	38	21	40	32	23	1
13 $\frac{3}{4}$	829	2	1	6	32	9	6	1	2	3	11	16	8	2
14	845		2	9	2		1	1		1	4	2	1	
14 $\frac{1}{4}$	860			1	1		2	2		3	1	1	1	2
14 $\frac{1}{2}$	875		1				1	2		4	1			4
14 $\frac{3}{4}$	889			6	3		1	1	2	4		9	1	1
15	903		2		1					1		4		1
15 $\frac{1}{4}$	916			1	2					1	1	1		
15 $\frac{1}{2}$	929													
15 $\frac{3}{4}$	942								1					

suggested — represented by very few cells. In 18 dwarf mice of 8 weeks and older the binuclear cells make $7.1 \pm 0.9\%$ of all the cells measured.

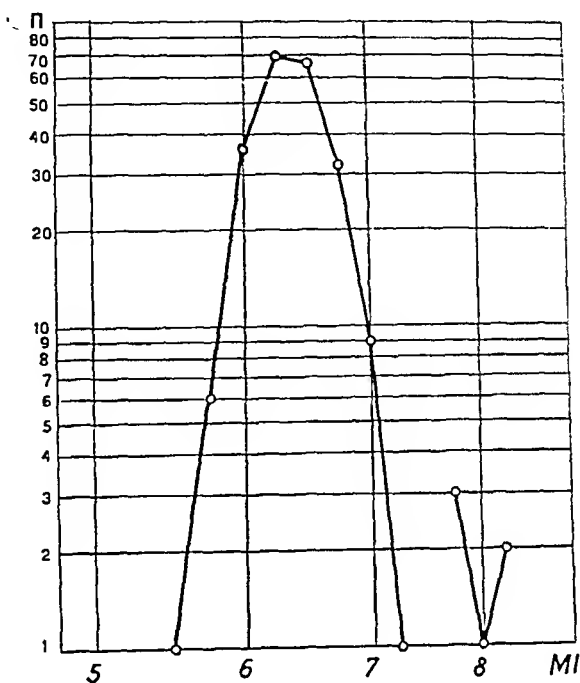


Fig. 2.

Nuclear diameters of dwarf mouse (1480).

The liver of 20 pituitary dwarf mice who were treated with pituitary growth hormone shows that after treatment with this hormone for $\frac{1}{2}$ —1 week the nuclear diameter distributions do not differ from those observed in untreated pituitary dwarfs. After 2 weeks' treatment, the mononuclear cells show a bimodal distribution, being composed of two nuclear classes, K_1 and K_2 . After about 3 weeks' treatment, a multimodal distribution is seen, made up of three single distributions — K_1 , K_2 and K_3 — and after about 7 weeks' treatment there is also a suggestion of a fourth nuclear class, K_4 (see Fig. 3). As mentioned, untreated dwarf mice show a single nuclear class of binuclear cells. After 2 weeks' treatment with growth hormone, however, we met with a bimodal distribution composed of two nuclear classes, $2K_1$ and $2K_2$. After 8—9 weeks' treatment a multimodal distribution appears, composed of three single distributions — $2K_1$, $2K_2$ and $2K_3$ — in a few cases with a suggestion of a fourth distribution, $2K_4$. The extreme parts of the compound distribution may show varying degrees of skewness due to components represented by few

observations. In 16 dwarf mice treated with growth hormone, aged 8 weeks or more, the binuclear cells make $14.2 \pm 0.9\%$ of all the cells measured.

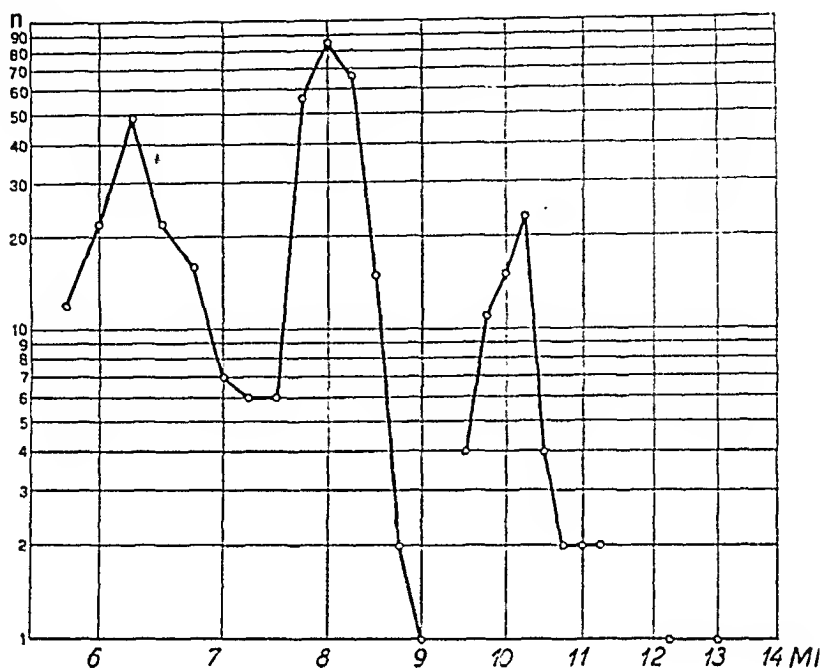


Fig. 3.

Nuclear diameters of dwarf mouse treated with pituitary growth hormone (1179).

Discussion.

All the examined specimens with pronounced nuclear class formation were essentially in harmony with the rule laid down by *Jacobj*. According to this rule, the ratio of the mean volumes is $1:2:4:8 \dots$, and consequently the logarithms of the mean diameter of the classes should be situated at a mutual distance of $\frac{1}{3} \log 2$ or 0.10034. In Table 1, for arithmetical reasons, the logarithms of the nuclear diameters are multiplied by 1000 (y) so that the maxima of the nuclear classes then would be situated at a mutual distance of 100.34. Table 1 shows that this approximately is the case.

In the present studies, individuals with lowered secretion of pituitary growth hormone show an abolished or greatly reduced formation of nuclear class series for mononuclear as well as binuclear cells in liver tissue, which normally develops several distinct nuclear classes. Also the formation of polynuclear (binuclear) cells is significantly decreased in pituitary dwarf mice, as in these animals only

7.1 ± 0.9 % of the liver cells are binuclear, whereas in normal control 16.2 ± 0.8 % of the liver cells are polynuclear. These figures are obtained from untreated material. On employment of *Pfuhl's* correction for determination of the real number of binuclear cells with allowance for the thickness of the section, etc., this difference will become even more pronounced.

On treatment of pituitary dwarf mice with growth hormone more nuclear classes will develop until the conditions no longer differ from the normal. Correspondingly, the number of binuclear cells increases to 14.2 ± 0.9 %, which does not deviate demonstrably from the normal. While it appears as if no previous investigations have been carried out on the nuclear size and nuclear class formation in individuals with decreased secretion of anterior pituitary growth hormone, *Lee* (1938) and *Lee & Freeman* (1940) have examined the liver cells in normal rats treated with pituitary growth hormone. But their investigations do not touch on the nuclear class formation. These authors mention that the effect of growth hormone extracts on rat livers is by way of cellular multiplication showing an increase in the mean diameter of the nuclei from $7.12 \pm 0.06 \mu$ for the controls to $7.50 \pm 0.04 \mu$ for treated rats with free access to food. The findings reported by *Lee* touch on the problems in the present work only insofar as the increase in the nuclear class formation under the growth of the mouse liver naturally implies an increase in the mean diameter of the liver cells as described by *Lee* — that is, if the corresponding conditions in mouse and rat be comparable.

What significance may then be assigned to the pituitary growth hormone with regard to the nuclear class formation?

From the findings here reported the growth hormone appears to be a factor necessary to the formation of more nuclear classes, for these develop but defectively when the secretion of growth hormone is decreased, and when growth hormone is supplied to the animal, series of nuclear classes reappear. The cellular point of action for the growth hormone being unknown, however, it is not practicable from the present studies to decide whether the growth hormone as an eliciting factor stimulates the individual cells to growth, or whether it intervenes in metabolic processes of the cells (e. g., the protein metabolism) as an essential link in the building up of the cells.

From Tables 1 and 2 it will be noticed that the first nuclear class (K_1) in the liver cells of pituitary dwarf mice on an average shows lower values for the nuclear diameter than does K_1 in normal mice. In the graphs this observation corresponds to a shift to the left in the nuclear diameter distributions. Shifts in the location of the nuclear classes have been observed previously under different circumstances. Thus, *Caspersson & Holmgren* (1934) found that in mice the nuclei of the liver cells increased in size at a maximal glycogen content of the liver, and decreased at a minimal glycogen content; and

Benecke (1937) has reported similar findings in rabbits. In pituitary dwarf mice, however, the glycogen content of the liver cells is more abundant than in normal mice (*Mollenbach*, 1941), on which account the shifting to the left of the nuclear classes hardly may be explained as connected with the glycogen content of the liver cells. *Wernel & Ssinewa* (1934) think they have been able to show that nitrogen deficiency brings about a shift to the left in the location of the nuclear classes in the rat liver, whereas, conversely, a high protein diet (*Schröter*, 1937) is said to shift the nuclear classes to the right. These experiments are too few, however, to allow us to explain the demonstrated shift to the left of the nuclear classes in pituitary dwarf mice as signifying a disturbance of the protein metabolism of the liver cells, but they still suggest some guiding lines for further studies on the cytological effects of the anterior pituitary growth hormone.

Summary.

In 27 normal mice, 27 pituitary dwarf mice and 20 dwarf mice treated with pituitary growth hormone it is shown that the formation of nuclear class series and polynuclear cells in the liver ceases or is reduced markedly by a decrease in the secretion of anterior pituitary growth hormone.

Supply of purified growth hormone to individuals with decreased growth hormone secretion induces development of nuclear class series and polynuclear cells until these features no longer differ from the normal.

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ON THE SIZES OF THE NUCLEI IN THE GLANDULA INFRAORBITALIS OF THE WHITE RAT

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Glandula infraorbitalis is a lacrimal gland lying in the outer angle of the orbit in a number of rodents. This gland and the glandula orbitalis externa which is situated in front of the external acoustic duct and the parotid gland, have a joint duct emptying into the conjunctival sac. Animals in whom these glands occur lack the lacrimal gland which is otherwise typical of the mammals.

This gland in the rat was first described by *Loewenthal* (1895) as a yellowish brown organ resembling a stomach laterally compressed, and situated partly behind and partly above the zygomatic arch. It is separated from the skin and the subcutis by a firm fibrous membrane running from the fascia temporalis and the upper edge of the zygomatic arch.

During my investigations of nuclear class and cell division conditions in the outer orbital gland in the white rat (*Teir*, 1944), I noticed that intentionally or unintentionally this gland had been to a fairly large extent subject to the interest of investigators, whereas the glandula infraorbitalis since the time of *Loewenthal* had hardly been investigated at all. As various kinds of gland cells, exhibiting great variations in the size of the cells and the nuclei, were further found in the organ, I considered that an investigation in the statistics of variation of the size of the nuclei might be of interest.

I wish to express my gratitude to Professor *Osmo Järvi* (the Finnish University at Åbo) under the guidance of whom I carried out the mentioned work on the outer orbital gland, for the advice he gave me while I completed also the present work.

Table 1.

Variations in the nuclear sizes in three adult rats.

Nuclear diameters in μ	r 29 (4 months old)			r 44 (5 months old)		
	Number of mononucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei.	Number of double-nucleated cells	Number of mononucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei.	Number of double-nucleated cells
5.50	2			1		
5.75	6			5		
6.00	2	$\left. \begin{array}{l} D_1=6.11 \\ K_1=228 \end{array} \right\}$		9	$\left. \begin{array}{l} D_1=6.13 \\ K_1=230 \end{array} \right\}$	
6.25	6			10		
6.50	4			4		
6.75	2			5		
7.00	4		1	2		2
7.25	11		3	7		3
7.50	19	$\left. \begin{array}{l} D_2=7.71 \\ K_2=458 \end{array} \right\}$	5	26	$\left. \begin{array}{l} D_2=7.76 \\ K_2=467 \end{array} \right\}$	9
7.75	25		7	36		14
8.00	15		1	29		5
8.25	7		2	9		2
8.50	1		2	3		
8.75	1			1		1
9.00	4			3		
9.25	9		2	6		2
9.50	12	$\left. \begin{array}{l} D_4=9.65 \\ K_4=896 \end{array} \right\}$	10	6	$\left. \begin{array}{l} D_4=9.84 \\ K_4=953 \end{array} \right\}$	9
9.75	14		7	18		11
10.00	9		4	15		5
10.25	6			6		2
10.50				4		
10.75	2					
11.00				1		1
11.25	2			1		
11.50			1			
11.75	1		1			
12.00	1	$\left. \begin{array}{l} D_8=12.47 \\ K_8=1951 \end{array} \right\}$			$\left. \begin{array}{l} D_8=12.87 \\ K_8=2131 \end{array} \right\}$	2
12.25	1		2	1		1
12.50	2		2			1
12.75	3		2			
13.00	1			1		
13.25			1	1		
13.50				1		
13.75						
14.00						
14.25				1	$\left. \begin{array}{l} D_{16}=14.69 \\ K_{16}=3170 \end{array} \right\}$	
14.50						
14.75				2		
15.00				1		1
15.25						
15.50						
15.75			1			
16.00	1					
—						
18.25	1	$\left. \begin{array}{l} D_{32}=18.75 \\ K_{32}=6603 \end{array} \right\}$				
18.50						
18.75						
19.00						
19.25	1					
	175	A total of 229 cells, of which 76.41% mononuclear cells and 23.59% binuclear cells	54	215	A total of 286 cells of which 75.17% mononuclear cells and 24.83% binuclear cells	71

Nuclear diameters in μ	r 86 (16 months old)			r 29, 44 and 86 together		
	Number of mononucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei.	Number of double-nucleated cells	Number of mononucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei	Number of double-nucleated cells
5.50				3		
5.75	4			15		
6.00	8	$\left\{ \begin{array}{l} D_1=6.17 \\ K_1=235 \end{array} \right.$		19	$\left\{ \begin{array}{l} D_1=6.16 \\ K_1=234 \end{array} \right.$	
6.25	16			32		
6.50	5			13		
6.75	4			11		
7.00	3			9		3
7.25	7			25		6
7.50	17	$\left\{ \begin{array}{l} D_2=7.85 \\ K_2=484 \\ (470) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_1=7.82 \\ 2K_1=478 \end{array} \right.$	7	62	$\left\{ \begin{array}{l} D_2=7.76 \\ K_2=467 \\ (468) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_1=7.72 \\ 2K_1=460 \end{array} \right.$	21
7.75	44		13	105		34
8.00	33		6	77		12
8.25	14		3	30		7
8.50	9		1	13		3
8.75	3			5		1
9.00	1			8		
9.25	4			19		4
9.50	3	$\left\{ \begin{array}{l} D_4=9.75 \\ K_4=927 \\ (940) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_2=10.22 \\ 2K_2=1065 \end{array} \right.$	3	21	$\left\{ \begin{array}{l} D_4=9.77 \\ K_4=933 \\ (936) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_2=9.73 \\ 2K_2=921 \end{array} \right.$	22
9.75	14		15	46		33
10.00	6		6	30		15
10.25	7		5	19		7
10.50				4		
10.75			1	2		1
11.00	2		1	3		2
11.25	1			4		
11.50						1
11.75				1		1
12.00				1		
12.25	1	$\left\{ \begin{array}{l} 2D_4=12.89 \\ 2K_4=2741 \\ (1880) \end{array} \right.$	1	3	$\left\{ \begin{array}{l} D_8=12.53 \\ K_8=1966 \\ (1872) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_4=12.50 \\ 2K_4=1953 \end{array} \right.$	5
12.50			2	3		5
12.75	1		2	4		5
13.00				1		
13.25			2	1		3
13.50				1		
13.75						
14.00						
14.25				1		
14.50						
14.75				2	$\left\{ \begin{array}{l} D_{16}=14.80 \\ K_{16}=3294 \\ (3794) \end{array} \right. \quad \left\{ \begin{array}{l} 2D_8=15.25 \\ 2K_8=3546 \end{array} \right.$	1
15.00	1	$\left\{ \begin{array}{l} D_{16}=15.38 \\ K_{16}=3644 \\ (3760) \end{array} \right.$		2		
15.25						
15.50						
15.75	1			1		1
16.00				1		
18.25			1	1	$\left\{ \begin{array}{l} D_{32}=18.75 \\ K_{32}=6609 \\ (7488) \end{array} \right.$	1
18.50						
18.75						
19.00						
19.25				1		
	209	A total of 278 cells of which 75.18% mononuclear cells and 24.88% binuclear cells	69	599	A total of 793 cells of which 75.54% mononuclear cells and 24.46% binuclear cells	194

Material and methods of investigation.

A total of 31 white rats were examined the majority of which were used for investigations of the so-called *Harder's* cells, while three animals only were used for determining nuclear classes in the usual glandular portions. All three were adult animals, one was 4 (rat 29), one 5 (rat 44) and one 16 (rat 86) months old. Further details of these experimental animals are given in table 1, p. 28 of the above mentioned work. The methods of investigation in determining the nuclear classes are the same as in that work.

Nuclear sizes of the gland cells.

Glandular tissue which to its structure completely corresponds to the tissue in the outer orbital gland, is found in the inner orbital gland. *Loewenthal* considers these glands to be different parts of one and the same gland. With regard to the more delicate histological structure of the inner orbital gland I therefore beg to refer to earlier investigations (*Loewenthal* 1895, 1900, 1926/27), *Guicysse-Pellesier* 1923, *Järvi* 1938).

Already a superficial examination of the nuclear sizes of the cells in the glandula infraorbitalis of the rat shows that these sizes vary to a very large extent. The cells are distinctly defined. The nuclear membrane appears clearly in the preparations fixed in *Bouin's* fluid and stained in *Heidenhain's* hematoxylin.

The result of the measuring of the nuclei appears from table 1. Here as in the outer orbital gland of the rat we find distinct frequency maxima for the nuclear volumes.¹⁾

The smallest nuclear class has an average diameter (D_1) of $6,16 \mu$ corresponding to an average of $234 \mu^3$. As this value was obtained through determining the diameter of rather a small number of nuclei, and being, on the other hand, the base for determining the theoretical values for the volumes of the other nuclear classes, I considered a more thorough calculation of this average value necessary, although the obtained values for the three animals were rather close to each other. For this purpose a special determination of the size of the smallest nuclei was made, about 100 nuclei being drawn separately for each animal (table 2). Since the average diameter ($6,12 \mu$) thus obtained differed from the first mentioned value by 0,04 only, it should be considered reliable.

1) A more detailed description of the arrangement of the tables of variance will be found in my paper on the nuclear sizes in the outer orbital gland of the rat (*Teir*, 1944, p. 42). The diameter values for cells with two nuclei are theoretical, and have been included in order to facilitate a comparison with the mononuclear cells. They are the third root of the value received through addition of the values of the two nuclear volumes in a cell with two nuclei.

The second nuclear class is here, as in the glandula extraorbitalis, the regular class of the organ, being formed both of mononuclear cells and cells with two nuclei (K_2 and $2K_1$).

The third nuclear cases is clearly represented in all animals and is also characterized by very evident frequency maxima.

Table 2.

Results of special measurements of the smallest cell nuclear in three adult rats.

Nuclear diameters in μ	r 29	r 41	r 88	r 29, 41 and 88 together
5.00				
5.25	3	1	3	7
5.50	8	3	8	19
5.75	19	14	14	47
6.00	34	25	38	97
6.25	29	38	44	111
6.50	12	13	16	41
6.75	3	4	3	10
7.00				
	98	98	126	322

The fourth class is represented by a smaller number of cells. Cells with two nuclei form frequency maxima in all animals. The two younger rats show weak maxima for the mononuclear cells while these cells do not form any evident maxima in the older rat, as was also the case with regard to two age groups in glandula orbitalis externa (Teir 1944, p. 68 and 80).

We have thus established that four distinct nuclear classes, composed both of mononuclear cells and cells with two nuclei, can be found in the inner orbital gland of the rat. Already in his first description of this gland, Loewenthal (1895, p. 126) says that nuclei measuring 8—10 μ , 11.5—17 μ , 16—23.5 μ and 23.5—26.4 μ in diameter occur in the gland, thus actually stating that four nuclear classes occur in this gland. Besides the four nuclear classes we shall find in table 1 also a minor number of larger nuclei forming indistinct frequency maxima. Because of the small number of these cells I considered special measuring of the largest nuclei in the preparations necessary in order to find out whether these large cells also belonged to certain nuclear classes.

For this purpose I examined three approximately equally large sections of each animal, drawing the contours of the largest mononuclear cells and cells with two nuclei occurring in the preparation. This investigation was much hampered by the fact that the large

nuclei are frequently irregular in form and consequently not suitable for determining the volume. The values obtained are thus not quite as reliable as in the former determination of the volumes, and I have therefore entered the frequency values for the diameters in a table of variance (table 3) with a difference of 0.5μ between the variants. I have included in the table the values from 14μ and upwards. This table does not show as distinct frequency maxima as in the former measurements.

However, we find in the 4 months old rat distinctly two higher nuclear classes besides the four classes so far found in the organ. Both the fifth and the sixth class are represented by mononuclear cells and by cells with two nuclei the average volume value of which comes close to the theoretical values for these classes.

In the 5 months old rat only the cells with two nuclei form fairly distinct tops while the tops are very indistinct in the mononuclear K_{16} and non-existent in the K_{32} .

In the 16 months old rat large cells are not found to the same extent as in the two younger experimental animals. However, a frequency maximum for the cells with two nuclei belonging to the fifth nuclear class, also occurs here while the mononuclear cells form no maxima.

In the summary of the diameter values for the nuclei in the last column of the table, we find distinct tops for the cells with two nuclei while the mononuclear cells do not form quite as distinct maxima, probably because of the great variations in the form of these cells.

From the special measurement of the nuclei of the large cells appears, however, that these form nuclear classes in the same way as the smaller cell nuclei.

It can be seen from table 1 that the obtained average volume values for the different nuclear classes correspond well with the values received through multiplication of the lowest value with 2, 4, 8 and 16. In order further to elucidate this circumstance we shall determine the relation between the averages given in the last column of table 1 using, however, for the fifth and the sixth nuclear classes the averages obtained by special measurement of the largest cell nuclei.

For the mononuclear cells we obtain in this way the following series:

$$K_1 : K_2 : K_4 : K_8 : K_{16} : K_{32} = 234 : 467 : 933 : 1966 : 4096 : 6904 = \\ 1 : 1.99 : 3.98 : 8.36 : 17.08 : 29.19,$$

and for cells with two nuclei this series:

$$2K_1 : 2K_2 : 2K_4 : 2K_8 : 2K_{16} = 460 : 921 : 1953 : 4291 : 6591 = \\ 1.92 : 3.94 : 8.34 : 16.20 : 28.16.$$

We find that the relation of the volumes both of mononuclear cells and cells with two nuclei in the different nuclear classes, the possibility of errors in the measurement taken into consideration, is

Table 3.
Results of special measurements of the largest cell nuclei in three adult rats.

Nuclear diameters in μ	r 29 (4 months old)			r 44 (5 months old)			r 86 (16 months old)			r 29, 44 and 86 together		
	Number of mono-nucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei	Number of double-nucleated cells	Number of mono-nucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei	Number of double-nucleated cells	Number of mono-nucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei	Number of double-nucleated cells	Number of mono-nucleated cells	Frequency maxima of the nuclear sizes in cells with one and two nuclei	Number of double-nucleated cells
13.50	8		1	5		1	2			10		2
14.00	1		5	5		1	2			8		1
14.50	2		7	6		1	1			10		6
15.00	3		7	7		4	1			9		14
15.50	7	$D_{10} = 16.00$ $K_{10} = 4096$ (3648)	4	7	$2D_8 = 16.06$ $2D_8 = 16.06$ $2K_8 = 4114$ (3680)	7	1	$2D_8 = 16.12$ $2K_8 = 4189$ (3760)	3	15	$D_{10} = 16.00$ $K_{10} = 4096$ $2D_8 = 16.25$ (3744) $2K_8 = 4291$	14
16.00	2	$2D_8 = 16.44$ $2K_8 = 4435$	8	1		6	1		1	4		15
16.50	2		1	4		1				6		1
17.00	2		3	1		1	1			4		4
17.50	2		3	2		3				2		3
18.00	4	$D_{32} = 19.04$ $K_{32} = 6904$ (7296) $2D_{10} = 19.25$ $2K_{10} = 7153$	2	1	$2D_{10} = 18.80$ $2K_{10} = 6332$ (7360)	4			1	5	$D_{32} = 19.04$ $2D_{10} = 18.75$ $K_{32} = 6904$ $2K_{10} = 6591$ (7488)	7
18.50	2		4	3		3				3		7
19.00	5		3	1		1				6		3
19.50	4		3			1				1		3
20.00	1		1			1				1		1
20.50										1		1
21.00												
21.50												
22.00												
22.50												
23.00												
	35		33	42		32	8		5	85		78

1:2:4:8:16:32 which proves that they follow *Jacobj-Heidenhain's* rule of growing.

Comparing the measurements of the nuclei of the inner orbital gland of the rat with those performed by me of the outer orbital gland of the same animal, might be of interest since these organs are so closely related, the former having arisen through a protrusion of the chordlike lumenless bud of the latter, (*Loewenthal* 1912, *Teir* 1944, picture 8, p. 181).

If, in this comparison, we pay attention first to the nuclear diameters of the two glands we shall find that these coincide to a high degree which appears most clearly in comparing the values given in table 1 to the corresponding figures for the *glandula orbitalis externa* (*Teir* 1944, table 21, p. 97).

As regards the nuclear classes, on the other hand, we find four distinct classes in both glands in adult animals. There are, however, a greater number of large cells and nuclei in the *glandula infraorbitalis* than in the *glandula extraorbitalis* which already appears from table 1, but still better by a comparison of the special measurements of cells with large nuclei in the two organs (table 3 in this work and table 20, p. 93 in the paper on the extraorbital gland). The fifth nuclear class appears vaguely already in the ordinary determination of the nuclear classes, and is quite marked in the special determination, whereas this nuclear class in the outer orbital gland can be found only by special measuring. The sixth nuclear class is not represented at all in the outer orbital gland but occurs, although very vaguely, in the inner orbital gland.

The reason why in two organs so alike in structure as the inner and outer orbital glands, we find more and higher nuclear classes in the one than in the other, might depend on the varying mechanical pressure conditions described by *Järvi* (1938), to which the glands are subject from their immediate surroundings during the period of development. By increased pressure a complete division of the cells and the acini would be suppressed leading to the occurrence of incomplete forms of division and polymerization of the nucleus. The *infraorbital* gland lies jammed in the outer angle of the orbit and is further covered by a firm membrane, whereas the *extraorbital* gland lies free in front of the external acoustic duct and the parotid gland, and is covered only by its own capsule and the skin. This fact speaks in favour of *Järvi's* hypothesis.

That six different nuclear classes occurred simultaneously in the inner orbital gland is quite a remarkable incidence since, as far as I know, so many nuclear classes have not been found in other normal organs. True, large cell nuclei, up to 16 and 32 times the smallest cell volume, have been found (*Jacobj* 1935, *G. Hertwig* 1942) in the central nervous system but they have been calculated with a basic unit of the smallest nuclear class volume, established by *Jacobj* in the

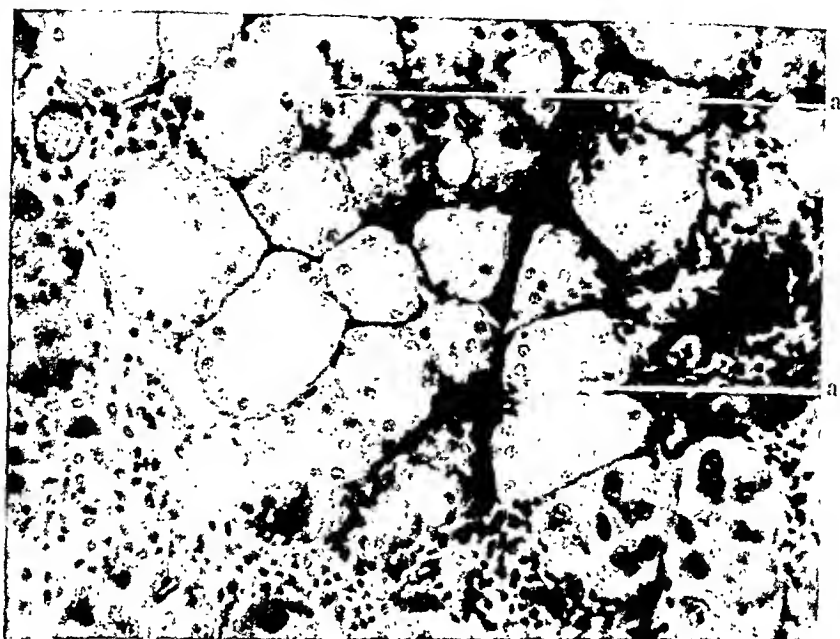


Fig. 1.

The light cells in the centre are *Harder's* cells forming acini with large lumina; in places traces of a tubular structure (a). Rich lymphocyte infiltration. The rat over 1 year old. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 250$.

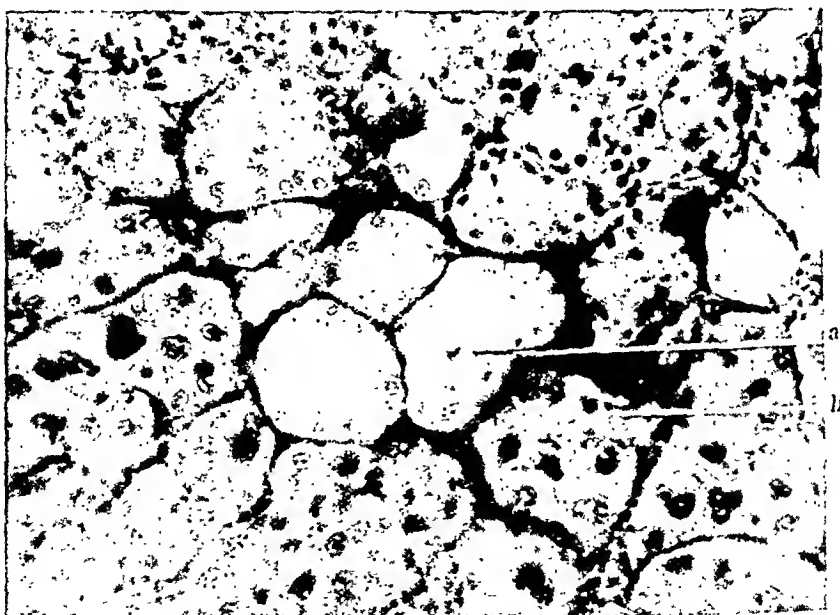


Fig. 2.

Harder's cells, mostly forming acini with narrow lumina. Mitoses are seen both in these cells (a) and in the adjacent ordinary gland tissue (b). Fairly rich lymphocyte infiltration. Same action as in Fig. 1. Magnification $\times 250$.

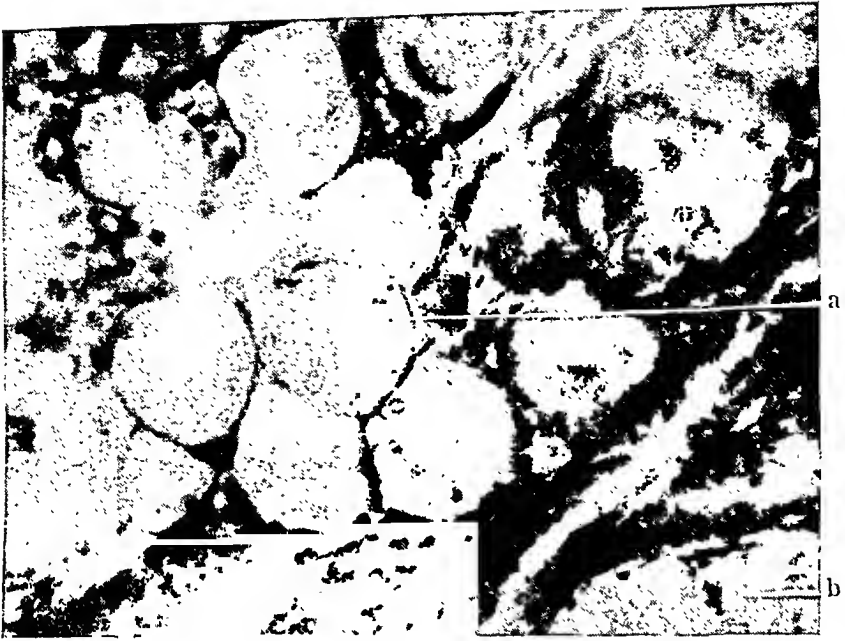


Fig. 3.

A beautiful rosetted tubular gland complex in *Harder's* gland portion of the same preparation as in the two foregoing figures. Mitoses are here found both within (a) and outside (b) this cell group. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 350$.

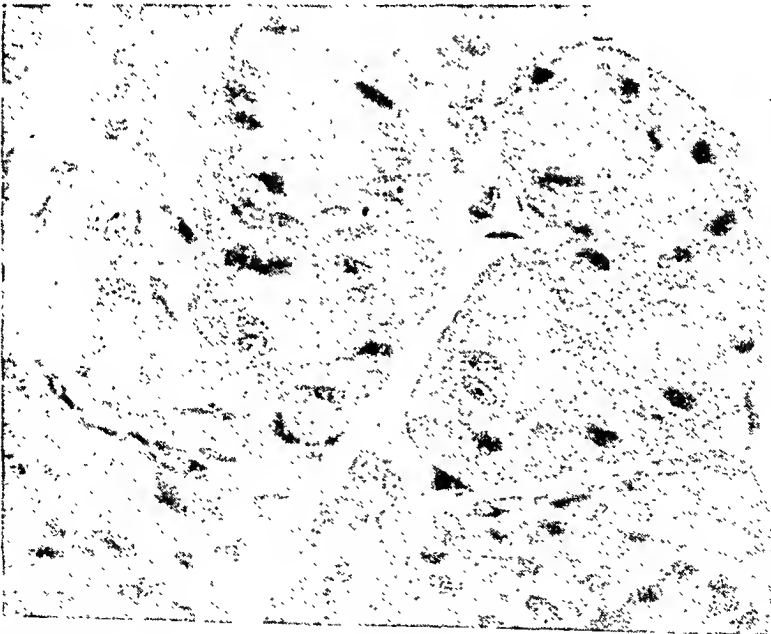


Fig. 4.

Deviating, small and irregular cells forming a separate group in a rat of 4 months. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 500$.



Fig. 5



Fig. 6



Fig. 7

Fig. 5.

A »reconstruction nucleus«, the size of $21,5 \times 19 \mu$, in a rat of 4 months. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 1000$.

Fig. 6.

A nucleus the size of $21 \times 14 \mu$ with a nucleol of $12 \times 9,3 \mu$ in a rat of 4 months. Just above the large nucleol a small nucleol in the same nucleus is seen. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 1000$.

Fig. 7.

A large cell with three large and dented nuclei in a rat of 5 months. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 1000$.



Fig. 8



Fig. 9

Fig. 8.

A cell with four nuclei in a rat of 1 year. The two uppermost nuclei have an average diameter of 12.25 and 11.75 μ and belong to the fourth nuclear class, whereas the two lowest nuclei with an average diameter of 9.59 and 8.75 μ should be referred to the third nuclear class. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 1000$.

Fig. 9.

Nuclear substance the length of 29 μ , comprising a formation resembling amitosis. In another projection two different nuclei are, however, clearly distinguishable the limits of which are indicated by the arrow in the figure. *Bouin-Heidenhain's* iron hematoxylin. Magnification $\times 1000$.

liver of the mouse. Actually they form but three nuclear classes.

Beside the cell species mentioned in the foregoing, *Loewenthal* also described various deviating cell species in the outer orbital gland of the rat, and we shall in the following first investigate the types which occur dispersed among the cells in the above mentioned more solid portions of the gland.

Very large, actually giant cells with irregular and dented nuclei were described by *Loewenthal* already in his first work of 1895 and compared to the megakaryocytes of the bone marrow. As already pointed out, the largest cell nuclei in the inner orbital gland are often irregular in form. There are clefts in the nucleus which sometimes gives it an amitosis-like appearance. Closer examination generally reveals, however, that a nucleus of this kind is often composed of two nuclei pressed against one another in a cell with two nuclei, usually covering each other in the section. The largest nucleus measured by me had an average diameter of 21 μ , thus actually a »giant cell«.

In the larger cells the nucleoli are sometimes of an imposing size (picture 6), or number. Large cells, resembling reconstruction mitoses (*Teir* 1944, p. 135—138) are occasionally found, and picture 5 shows a nucleus of the size of $21.5 \times 19 \mu$ and belonging to the sixth nuclear class. Polynuclear cells are frequently found. 3—6 nuclei in one cell is not a rare occurrence. The nuclei are often clustered together in a heap which renders it difficult to determine exactly their number in the sections. A very large cell with three nuclei lying close together is found in picture 7. This cell is from the 4 months old rat, and resembles at a cursory glance an amitosis. Picture 9 shows two longish nuclei close to one another resembling an amitosis. However, the arrow points at a distinct limit between two nuclei which can be observed in the microscope in another projection. The two small nuclei in the lower part of the picture represent the smallest nuclear class. Picture 8 shows a cell with four nuclei, two of which have a size of about 12 μ and two of about 9 μ . Deviating cell forms of this kind are not seldom found in the inner orbital gland of the rat.

Loewenthal (1900, p. 540) also described a type of deviating cells with remarkably small nuclei and acini with narrow lumina. Like *Loewenthal* I also believe that these cells are young *Harder's* cells which will be dealt with more closely in the following.

I wish to describe briefly one more type of deviating cells. Lobuli and lobes comprising small, often irregular nuclei, staining intensely in hematoxylin, and sometimes exhibiting ragged contours, are occasionally found in the gland. Such a portion can be seen on picture 4. The nuclei are intensely stained with *Feulgen's* nuclear stain particularly in *Bouin*-fixed preparations whereas *Champy's* fixation apparently was not equally suitable for *Feulgen's* reaction. The irregular form of the nuclei excludes every possibility of determining the nu-

clear sizes. An approximative estimation of the nuclear sizes would indicate the lowest nuclear class. It is also possible that the latter have the power of developing later in another direction.

The so-called Harder's cells and the size of their nuclei.

The compact gland portions described above mostly dominate the histological picture. However, *Loewenthal* (1895) described a deviating cell type in the inner orbital gland of the rat, viz. the so-called *Harder's cells*.

These cells resemble the cells in *Harder's gland*, also situated in the orbit. The cells are light with small nuclei of almost equal size and forming acini with remarkably large lumina which in turn form gland portions of varying sizes which occur in groups in the organ. Similar gland cells were described by *Collin* and *Florentin* (1930), *Järvi* (1938) and *Teir* (1944) in the outer orbital gland of the rat.

I did not, however, find such cells in the above mentioned 3 rats, although sections were taken at different depths of the paraffin blocks. *Järvi* also mentioned that in several rats he did not find this kind of cells in the outer orbital gland. When the outer orbital gland was described for the first time by *Loewenthal* he stated that he found no such cells in the organ.

Since the occasional absence of one type of cell from an organ seemed somewhat strange to me, I examined 17 white rats in order to establish whether *Harder's cells* occurred or not. The ages of 3, 4, 5, 8 and 9 months were represented by 3 animals and the age of 1 year by 2 animals. All were of the same origin as the former animals. I made on an average 11 sections at different depths of the paraffin blocks both of the left and the right inner orbital gland. Cells resembling *Harder's cells* were found only in two animals, in a male of 5 months and in another over 1 year old.

The latter proved to be rare specimen for examination since *Harder's cells* occurred so numerously that they were often predominating in the histological picture. They occur in pronounced groups (picture 1 and 2), one section showing groups of 50—60 acini. They are not covered by a capsule but are directly adjacent to the acini in the compact gland portions and, as to the localization in the organ, they do not seem to follow any definite rules. The size of the acini is about the same as in the compact gland portions. The lumina are mostly quite large. In *Bouin*-fixed preparations stained with *Heidenhain's iron hematoxylin*, they contained a secretion, stained greyish, in which solitary desquamated epithelial cells can be seen. The cells are flat but may sometimes be cubic. The cell limits are not nearly as well defined as in the compact gland portions.

The nuclei are small, often somewhat irregular, but vary very little in size. Table 4 gives the result of the measuring of the nuclei. Here

as in the outer orbital gland (*Teir* 1944) is but one nuclear class, the average volume value of which (246) comes rather close to the average (234) of the smallest nuclear class in the compact gland portions. The extent of the variation maximum is due to the frequently oval form

Table 4.

Variations in the nuclear sizes of the so-called Harder's gland cells.

Nuclear diameters in μ	Number of mono-nucleated cells	Frequenzy maxima of the mononucleated cells
5.25	8	$\left. \begin{array}{l} D_1=6.27 \\ K_1=246 \end{array} \right\}$
5.50	19	
5.75	30	
6.00	67	
6.25	41	
6.50	24	
6.75	20	
7.00	15	
7.00	11	
7.25	4	
7.50	1	
7.75		
8.00		
8.25	1	
8.50	1	
8.75		
9.00	1	
9.25	1	
9.50		
	244	

of the cells and also to the fact that they often seem somewhat swollen, sometimes even showing signs of karyolysis. I found only one definitely doublenucleated cell. The 4 large nuclei occurring in the table, and coming close to the volume value of the third nuclear class, lie just on the limit of the compact gland portions.

Also in the 5 months old rat the nuclei of *Harder's* cells are of equal size and belong to the lowest nuclear class.

In order quickly to obtain a lucid picture of the relations of the nuclear classes I have on a paper drawn circles with diameters corresponding to those of the average volume values of the nuclear classes earlier determined in the organ. I filled these circles with black Indian ink, cut them out and attached white paper handles to them. Moving these figures on the projection table and comparing them with the nuclear sizes in the preparation, and approximate idea is obtained of the relations of the nuclear classes in the section.

Both in the outer and the inner orbital gland small light dots, hardly distinguishable to the naked eye, can sometimes be found on the surface. The idea occurred to me that these dots might correspond to the fatty *Harder's* cells, and in order to check this, I examined further 11 rats out of which 5 were four and 6 five months old. With the aid of a loupe both glands were freed on both sides and the surfaces were thoroughly examined also with the aid of a loupe. It appeared that all the glands in seven animals were homogenously yellowish brown and showed no spots. In 4 animals, however, light dots were found. In three animals these dots, of a size of 0.5—1 mm, were freed with the aid of a loupe and fixed in 10 per cent formalin. In the beginning of the fixation they were stained during 10—15 seconds in an ordinary *Giemsa* basic solution in order to be more easily recognized during the later treatment of the preparations. The preparations embedded in paraffin were stained with hemalum-eosin.

A total of 6 samples were taken from the three experimental animals, 4 of the inner and 2 of the outer orbital gland. *Harder's* cells were found in 5 samples. As to the sixth sample it was already during the preparation uncertain whether the extremely small dot was included in the microscopic preparation. This should prove, considering that these cells are generally rare in the two lacrimal glands of rodents, that the light dots on the surface of the gland correspond to *Harder's* cells.

Rather large, ramified tubular gland complexes are sometimes found in *Harder's* gland portions. These complexes occur independently of the age of the animals, being found also in the over one year old rat (picture 1, 2, 3). In these gland complexes mitoses are sometimes seen as also in adjacent compact gland portions (pictures 2 and 3). In places with more abundant regeneration of glandular tissue lymphocyte infiltrations may often occur (pictures 1 and 2). These observations indicate that *Harder's* gland portions either develop late, or that they may be able to continue their development long after the growth of the organ and the individual have ceased.

Summary.

1. In the inner orbital gland of the rat six different nuclear classes are found, the two highest, however, being comparatively weakly represented. The relation of the nuclear classes are as 1 : 2 : 4 : 8 : 16 : 32 following *Jacobj—Heidenhain's* rule of the doubling growth of the cells.

2. The small white dots sometimes occurring on the surface of the orbital glands in the white rat correspond to the so-called *Harder's* cells which may still develop long after the growth of the organ and the individual have ceased. These cells which form but one nuclear class, are more closely described.

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AN INCOMPLETE AGGLUTININ RELATED TO THE L-(LEWIS) SYSTEM

By P. H. Andresen & Kell Jordal.

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For both the Lewis^{1, 3} and the Lutheran² blood group systems the symbols L₁ and L₂ have been used. Therefore the following notation will be used.⁴ Gene: L₁ = Le^a, L₂ = Le^b and the phenotypes: L₁ + L₂ — = Le(a + b—), L₁ — L₂ + = Le(a—b+), L₁—L₂— = Le(a—b—).

The L-system is characterized by the receptors Le^a and Le^b, (Andresen 3)4)5), and in adults persons we find 3 phenotypes (Le(a+b—), Le(a-b+), Le(a-b—)) The heriditance is determined by two allele genes, Le^a and Le^b where Le^b is dominant. This theory alone does not explain the type (Le(a-b—)).

In the investigations with many anti-Le^a sera (from persons of type Le(a-b—)) we found the characteristic reaction with Le^a, but after some time a weak reaction was also obtained by most other blood cells, even if they have no Le^a-receptor.

The reactions were always very weak and the results were not significant, but we never found an agglutination with blood cells of type Le(a-b—). Recently we found a new anti-Le^a serum (Petra) from a mother of type A₁Le(a-b—). The weak reaction was here more marked, and after a few investigations it was possible to establish that the serum contained partly an anti-Le^a agglutinin, partly an incomplete agglutinin which reacted with about 90 per cent of all blood samples.

By using blood cells suspended in their own plasma or in albumin we got a very strong agglutination.

Table 1 shows the result of 300 investigations with anti-Le^a, anti-Le^b and the new agglutinin (in this paper designated anti-X and the receptor X).

The most characteristic result is that all X- are also (Le(a-b—) and in the case of types O and A₂ all Le(a-b—) are also X—.

Table 1.
Investigations with Anti-L₁, Anti-L₂ and Anti-X.

Blood Group	Reaction with Anti-		Reaction with serum »Petra«	
	Le ^a	Le ^b	+	-
Adults			51	0
O & A ₂	+	—	143	0
	—	+	0 (194)	24 (24)
	—	—		
A ₁	+	—	29	0
	—	+	33	0
	—	—	55 (117)	5 (9)
			(311)	(33)
Children				
O & A ₂			81 (81)	9 (5)
			54 (54)	4 (4)
A ₁			(135)	(9)

Table 2.

Blood sample		Serum	
Type	No.	»Petra« + Saline 1/1	»Petra« + albumin 1/1
Le (a+b—) X+	6	++	++
»	12	++	++
»	19	++	++
»	20	++	++
Le (a—b+) X+	3	(+)	++
»	10	—	++
»	13	—	++
»	14	—	++
»	17	—	++
»	22	(+)	++
»	23	—	++
»	28	—	+
Le (a—b—) X—	11	—	—
»	27	—	—

The difference between anti-Le^a and anti-X is demonstrated in table 2, but the difference will also be seen in other investigations: anti-Le^a reacts at 37° C., anti-X only at room temperature, and anti-Le^a is demonstrable by a specific hemolysin reaction.

It is more difficult to prove that anti-X is not anti-Le^b. The difference between anti-Le^b and anti-X will be 1) anti-X gives the same per cent X+ in group O and in group A₁, 2) and the anti-X gives also the same per cent X in children and adults (see table 1), 3) by ab-

sorption with Le(a+h-) cells all agglutinins anti-Le^a as well as anti-X will be absorbed.

Our investigations show that X must be an heritable property. It is therefore to be supposed that another factor x exist and that the homozygote (xx) inhibits the development of phenotypes of the Lewis-system.

Summary.

A new incomplete isoagglutinin is demonstrated which reacts with a heritable receptor found in 90 per cent of all persons. This receptor will be lacking in persons of type Le(a-b-) only.

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INVESTIGATIONS ON THE BLOODFACTOR P.

By K. Henningsen.

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In 1927 *Landsteiner & Levine* encountered a rabbit immune serum containing a hitherto unknown agglutinin, the corresponding blood-factor of which was designated P. (16). It was soon ascertained that the reactions of this agglutinin agreed to a certain extent with the reactions of an agglutinin found earlier in human serum, the so-called extraagglutinin I. Agglutinins reacting similarly were later found in normal animal sera especially from pigs, horses and cattle (20, 21). The reactions, however, of the sera derived from different sources, did not agree exactly (17) and consequently the different P-factors were designated in accordance with the sera used for the determinations as P_1 for P found by immune sera, P_n for normal animal sera and P_h for human anti-P sera. *Landsteiner & Levine* (18) found in Caucasians 81.9 per cent having the P-factor while in negroes 97.8 per cent were P positive. Another peculiarity was that the P-positive bloods were agglutinated in a greatly varying intensity and that the strongest reactions were most frequently met with in negroes. By means of family investigations it was made certain that P was a hereditary property but the exact mechanism could not be determined. (21, 19).

Later investigations on this blood-group have been somewhat hampered by the difficulties in obtaining potent anti-P sera. The immune sera could not be reproduced with any certainty, and the human anti-P sera were mostly too weak for grouping purposes.

Using normal animal sera (from pigs) *P. Dahr and coworkers* have performed extensive investigations (5, 6, 7, 8, 9, 10, 11), finding 74 per cent P+ in 7429 persons, and by family- and twin-investigations it was established that the P-factor was inherited as a Mendelian dominant depending upon a single pair of allelic genes P and p. In the

children, 4 exceptions from this hypothesis occurred, but illegitimacy could not be excluded in any of those cases.

Jungmichel (15) has tested 1586 persons finding 75,54 per cent P+. Among 40 families he encountered 2 families in which both parents were P—. None of the seven children in those families were P+.

By comparative determinations with a normal animal serum (cattle) and a human anti-P serum, *Andresen* (1) found a fairly good correlation, the percentages of P+ being 82,3 using the animal serum, 80,7 using human serum. The latter was a typical cold agglutinin reacting, however, specifically with P. By titration and absorption the material was divided into 3 groups according to the strength of the receptor. The distinction, however, between those groups was not sharply defined, there being apparently intermediary forms.

From *Statens Rättskemiska Laboratorium, Stockholm*, a series of mother-child combinations has been published supporting the above-mentioned theory of heredity (28, 14).

The authors investigations with regard to the determination of factor P, the distribution in the Danish population, the possible subgrouping according to strength, and the occurrence and characteristics of anti-P of human origin are presented in the following paper.

Technic.

It must be realised that the difficulties in P-grouping are greater than in grouping with the ABO- and MN-systems. On account of less potent sera the reactions are usually considerably weaker and require more time to be fully developed. On account of this, there is a greater risk of obtaining non-specific reactions, especially as dilution of test-sera after absorption or more intensive absorption, in order to increase the specificity, is prohibited by the low titer of the testsera. Another difficulty is that the distinction between P— and P+ is not sharp. There is an apparently gradual transition from P— through P-weak to P-strong corpuscles. L. & L. acknowledged this fact in their first communication on P and consequently designated some of the weaker reactions as P±. Dahr maintained to produce a sharp differentiation provided the testserum was of sufficient potency, but less potent sera necessitate a special and very delicate technic to attain this sharp differentiation.

Using animal sera the slide method has been preferred. Incubation usually has been performed at room-temperature and the reactions were read after 15 minutes (Dahr, Jungmichel) or with intervals of 5 minutes for 35 minutes in all (Jonsson). Andresen used the slide method with animal as well as with human serum, the reactions with the first being done at room-temperature, with the latter at 6 degrees centigrade. Further, absorption tests have been used to some extent (Jungmichel, Jonsson, Andresen) partly as a means of verifying weak receptors and partly for subgrouping according to receptor strength.

The technic of *Bengt Jonsson* from Statens Rättskemiska Laboratorium, Stockholm, is going to be described in some detail, the author's technic being influenced by that to a great extent. (13, personal communication). In routine P-grouping Jonsson employs normal animal as well as human sera. The reactions with the animal serum (horse) are done at controlled temperature, about 18 degrees centigrade, and microscopical and macroscopical readings are done every 5 minutes during 35 minutes. In this way an appraisal of the receptor strength is attained taking into consideration both the period until agglutination occurs, and the maximal strength of agglutination. Further the samples may be tested with anti-P serum of human origin, the tests being done in test tubes and read macroscopically after incubating overnight in the refrigerator. Finally every sample is checked by absorption with a human anti-P serum.

The author has used an absorbed normal horse serum as well as several human anti-P sera. The horse serum is absorbed undiluted with half a volume of P negative A, B and O erythrocytes the serum containing rather strong agglutinins against all 3 factors. After absorption and subsequent dilution with an equal volume of saline the serum reacts specifically against factor P provided that the readings are done not later than 30—45 minutes after mixing the serum and the corpuscles. This absorbed serum has a titer of 1:16 against strong P+ but in dilution 1:2 it usually gives distinct agglutination even with weak receptors. The weakest, however, give only microscopically visible agglutination. Unfortunately, the serum has some tendency of rouleaux formation, which, however, to some extent may be overcome by diluting 1:2 and by using very thin suspensions of corpuscles, about $\frac{1}{2}$ per cent in terms of sediment. During the first part of the investigation the human sera comprised an A₁ and a B serum called *Iris* and *Sine*. The anti-P agglutinins in each were relatively weak giving adequate reactions with the stronger receptors but especially in children they failed to react with the weak. It was not possible to absorb these sera with regard to Anti-A and anti-B as the anti-P titer by this absorption was further diminished on account of non-specific inhibition. The titer of both sera against strong receptors was about 1:16 by test on slide when read after about half an hour in the refrigerator. At room-temperature the sera agglutinated only the stronger and strongest receptors.

In the latter part of the investigation the human sera were employed exclusively by the testtube method, incubating the tubes in the refrigerator overnight. By this method serum *Sine* was found to have a titer of 1:64 against strong P+. In the course of the investigation several more human anti-P sera were encountered, of which a O-serum, *Leo*, had a titer of 1:64—128 using the testtube method, and further it was possible to absorb this serum with regard to agglutinins anti-A and -B, thus employing it for agglutination tests of all samples.

Occasionally another serum, *Gunnar*, group A₂, was used unabsorbed for testing A- and O-bloods. The testtube method in refrigerator necessitated removal of the non-specific coldagglutinins present in most sera, which was accomplished by absorption in the refrigerator overnight with half a volume of the sera's own cells.

During the first part of the investigation the actual tests with the horseserum were carried out on glass slides which were incubated in moist chamber at room-temperature for 30—45 minutes. The readings were done macroscopically and checked microscopically in order to prevent false positive readings due to rouleaux formation and further to detect the weakest reactions. Simultaneously the samples, with the exception of group AB, were tested against human sera, either *Iris* or *Sine*, according to group. The tests were made on slides incubated for about half an hour at 6 degrees centigrade and read quickly after removing the slide from the refrigerator. Here again the readings were made macro- and microscopically. In this way there might be some danger of obtaining false positive reactions on account of non-specific coldagglutination, but those reactions usually disappear after a couple of minutes at room-temperature, whereas the specific P-reaction is slightly more stable.

With this technic reliable results were obtained in a majority of cases but in a few they were dubious and not satisfactory. In consequence a more elaborate routine was developed on the lines of the Swedish technic, with which the author during a visit to Stockholm had the opportunity of familiarizing himself, and the last part of the investigation has been carried out by the following technic. Every sample is tested in 3 ways. First agglutination test with absorbed horse serum is carried out on slides at a temperature of 16—19 degrees centigrade. 2 drops of serum to one drop of the suspensions are used, and the reactions are read macro- and microscopically with intervals of 5 to 10 minutes until the final reading after 30 minutes. In this way the speed of the reaction is noted, and further the close scrutiny of all phases of the agglutination makes the final reading more certain. After testing with the horse serum every sample is titrated against the absorbed human serum, *Leo*, in dilutions 1:2—1:64. This titration is carried out in testtubes, which are incubated overnight in the refrigerator. The readings are made macroscopically after slight shaking. Finally a simplified absorption test is carried out by absorbing a dilution (1:6) of serum *Sine* with about one third volume of cells. After absorption overnight in the refrigerator the serum is tested against P+ of medium strenght by the testtube method. The final readings are made macroscopically after slight shaking, as well as by judging the configuration of the sediment in the bottom of the tube. The absorbing volume of corpuscles is 1 drop of tightly packed erythrocytes to 3 drops of serum. In the daily routine this may be attained by making the original suspensions contain just this amount

of cells. After using the suspension for the agglutination tests, it is centrifugated at 3000 rev. p. min. for about 10 minutes, the supernatant is removed and 3 drops of dilute serum are added to the sediment. As a matter of course a P-negative control is included in every absorption set-up, as well as a known positive control, preferably always the same, is included in every titration.

In this way every sample is checked 3 times with 3 different sera, and in most cases it is possible to reach a definite conclusion. In a few cases the agglutination reaction is very weak, and the simplified absorption does not confirm the agglutination with certainty. In such cases special absorption experiments are set up with both animal and human sera including known positive and negative samples. After absorption the sera are titrated against test bloods of different strength taking into consideration the speed of the reactions as well as the final titer. In a few cases of small children it is still impossible to reach a final decision, and these cases have to be referred to later examination, when the child has become older.

Results.

The first part of the investigation comprises 909 determinations in adults and 393 in children, of which 190 (20.9 per cent) adults and 96 (24.4 per cent) children were P—. All determinations are made with horse serum and a great proportion is checked by human anti-P serum. In a number of cases, especially children and the weakest receptors in adults, apparent discrepancies have occurred between horse and human sera, the reactions being positive with the former, negative with the latter. Those discrepancies may well be explained by the different potency of the test sera as indicated in the latter part of the investigation by absorption experiments. The latter part carried out by the more elaborate technic comprises 1436 adults and 540 children the P— frequency being 21.3 per cent (306) in adults and 25.9 per cent (140) in children.

In comparing the two parts of the investigation the agreement between the frequencies of P— and P+ is obvious, being well within the limit of the calculated standard error (29).

The formula of the standard error is:

$$\delta = \sqrt{p_o \times q_o \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}, \text{ } p_o \text{ and } q_o \text{ being the percentages of the observed}$$

frequencies of P— and P+ in the total investigation, n_1 and n_2 the number of observations in the 2 series. The standard error calculated in this way is:

$$\text{in adults: } \delta = \sqrt{21,1 \times 78,9 \left(\frac{1}{909} \times \frac{1}{1436} \right)} = 1,73,$$

$$\text{in children: } \delta = \sqrt{25,3 \times 74,7 \left(\frac{1}{393} \times \frac{1}{540} \right)} = 2,77.$$

The results of the entire material are summarized in tables 1, 2 and 3.

Table 1.
ADULTS

	total	percentage of material	P +	P —	percentage of P —
A:	1044	44.5	829	215	20.6 ± 1.25
O:	960	41	753	207	21.6 ± 1.33
B:	246	10.5	193	53	21.6 ± 2.68
AB:	95	4	74	21	22.0 ± 4.25
M:	700	29.8	533	167	23.8 ± 1.6
N:	508	21.7	407	101	19.9 ± 1.8
MN:	1137	48.5	909	228	20.0 ± 1.2
males:	1376	58.5	1080	296	21.5 ± 1.1
females:	969	41.5	769	200	20.6 ± 1.3
total:	2345	100	1849	496	21.1 ± 0.81

Distribution of P factor in adults within ABO and MN systems and according to sex.

Table 2.
CHILDREN

	total	P +	P —	percentage of P —
A:	398	301	97	24.4 ± 2.16
O:	364	266	98	27.0 ± 2.32
B + AB:	171	130	41	24.0 ± 3.36
total:	933	697	236	25.3 ± 1.4

Distribution of P factor in children.

It is seen from table 1 that the material represents an unselected sector of the population, the approximate normal frequencies in the Danish population being for A 43.5, O 42, B 10.5, AB 4, M 29, N 21.5 and MN 49.5 per cent (23, 24, 22, 3, 2). Further earlier statements (10, 30) are verified with regard to factor P's independence of the ABO and MN systems and sex.

The percentage of P— is found considerably less than the percentage attained by Dahr and coworkers, 26 per cent (7429 observations), Jungmichel, 24.46 per cent (1586 observations) and Jonsson 23.69 per cent (1089 observations). The difference between the author's and the Swedish percentages is not statistically significant and may be accounted for by the limited material. The percentage of Jungmichel is just inside the limit of 3 times the standard error of 1.36, while the percentage of Dahr is well outside this limit in comparing the two materials.

Table 3.

The frequencies of the different combinations within ABO, MN and P systems.

designation	ADULTS			CHILDREN
	number of observations	percentage	calculated percentage	number of observations
1 OMP+	201	8.60	9.62	85
2 OMP—	77	3.18	2.56	28
3 ONP+	161	6.85	7.15	51
4 ONP—	41	1.75	1.90	16
5 OMNP+	391	16.65	16.41	130
6 OMNP—	89	3.90	4.36	54
7 A ₁ MP+	196	8.35	7.78	63
8 A ₁ MP—	53	2.26	2.04	23
9 A ₂ NP+	143	6.10	5.69	43
10 A ₁ NP—	32	1.36	1.52	12
11 A ₁ MNP+	288	12.30	13.10	113
12 A ₁ MNP—	85	3.62	3.48	34
13 A ₂ MP+	48	2.02	2.29	24
14 A ₂ MP—	15	0.64	0.61	9
15 A ₂ NP+	36	1.53	1.70	19
16 A ₂ NP—	12	0.51	0.45	4
17 A ₂ MNP+	118	5.00	3.92	39
18 A ₂ MNP—	18	0.77	1.04	15
19 BMP+	63	2.68	2.40	20
20 BMP—	17	0.72	0.64	12
21 BNP+	51	2.17	1.78	17
22 BNP—	12	0.51	0.48	4
23 BMNP+	79	3.36	4.10	35
24 BMNP—	24	1.20	1.09	10
25 A ₁ BMP+	18	0.77	0.71	10
26 A ₁ BMP—	4	0.17	0.19	3
27 A ₁ BNP+	10	0.43	0.53	15
28 A ₁ BNP—	3	0.13	0.14	3
29 A ₁ BMNP+	24	1.20	1.21	19
30 A ₁ BMNP—	6	0.26	0.32	6
31 A ₂ BMP+	7	0.30	0.21	2
32 A ₂ BMP—	1	0.04	0.05	1
33 A ₂ BNP+	6	0.26	0.15	4
34 A ₂ BNP—	1	0.04	0.04	2
35 A ₂ BMNP+	9	0.38	0.35	8
36 A ₂ BMNP—	6	0.26	0.09	0
Total	2345			933

The calculations are based on the following percentages of frequency:
A₁:33.5, A₂:10, 0:42, B:10.5, A₁B:3.1, A₂B:0.9, M:29, N:21.5, MN:49.5,
P+:79, P—:21.

In order to investigate the reason of this discrepancy between the results of Dahr and those of the author's, the material of Dahr was subjected to a more thorough analysis. It is a conspicuous fact that the percentage of P— found by Dahr is growing gradually from 17,2 per cent in 1910 (6) to 24 per cent in 1941 (9) and to 26 per cent in 1942 (10). Taking the determinations of each communication separately, the material was then divided into groups, the author obtaining the respective figures by subtracting from the total number of the communication in question, the previously published number of determinations. It is seen that 1560 determinations in 1940 revealed 17,2 per cent P—. Later in 1940 an additional amount of 881 determinations was published the P— per cent in this communication being 34,6 (7). In 1941 (9) the P— percentage was found to be 25 in 1089 determinations, and in 1942 (10) 29,2 per cent P— was found in 2918 determinations. Even if the material consists of family and twin investigations to a great extent, and even if a considerable part of the samples originates from children, the findings strongly suggest a greatly varying potency of the sera used. Further the summing up of different series of investigations is hardly permissible, if the results differ to such extent, as it is found in those series. By calculating the standard error for the first and second series from 1940 the difference is found to be about 9 times the standard error, and a similar result is obtained by comparing the results of the first series of 1940 with the series of 1942.

Thus the discrepancy between Dahr's and the author's results may well be explained by supposing the use of less potent sera in Dahr's later investigations coupled with the inclusion of children in Dahr's figures. The P— frequency of 17,2 per cent, however, found by Dahr in the first series is not to be accounted for in this way, but the possibility of rouleaux formation simulating weak positive reaction can not be excluded, especially as it is not made clear, if microscopical control of the reactions has been performed.

The percentages of P— found by Andresen, 18,3 per cent in 506 observations and by Landsteiner & Levine in their original series, 18,1 in 265 observations, may well be explained by the limited number of observations.

Contrasting to the findings of Dahr (8) the percentages of P— in children in this investigation is found higher than in adults, and the reactions are generally weaker. This difference in frequency of the demonstrable P-factor is a little less than 3 times the standard error of 1.8, but nonetheless it is probably significant for the following reasons. On the whole, the difference has been constant during the whole course of the investigation. Further it is seen from table I. that the percentages of P— which in new born children is about 41.4 per cent with an additional 14.7 per cent not being positive with certainty, with one exception, which may well be due to chance, is higher than in adults until the age of 2 years, but it must be emphasized that the number of observations is limited, and the figures of the table is not considered a proof but just an indication.

Reliability of P-grouping.

As always in grouping work it is considered of the greatest importance to obtain clearcut and synonymous results especially if forensic use is aimed at. To a great extent the technic mentioned

Table 4.

age	total	P +	P —	P ±	percentage of P —	percentage of P ±
new borne	41	18	17	6	41.4	14.7
0— 2 months	53	38	14	1	26.4	1.9
2— 6 months	186	136	46	4	24.8	2.2
6—12 months	94	75	18	1	19.1	1.1
1— 2 years	59	42	17	0	28.8	0
more than 2 years	55	43	11	1	20.0	1.8
total	488	352	123	13	25.2	2.7

Distribution of factor P in children of different age groups.

above eliminates the difficulties which are encountered in telling the very weak P+ from P—. If agglutination with hetero- and homologous sera is very weak contingently absent, the absorption experiment may disclose a weak P-receptor. If the absorption is not satisfactory by the standard technic, the inhibition of the specific agglutinin may be demonstrated by special absorption experiments titrating the absorbed sera against P+ testbloods of various strengths. On the other hand a very weak agglutination by the horse serum which is suspected of being nonspecific may be confirmed as such by absorption experiments. Ordinarily no discrepancies are found between the results obtained with the various sera setting aside that the weakest receptors are not agglutinated by the homologous serum. In those cases the routine absorption mostly provides the conclusive evidence. Among 975 observations in adults 9 samples have been encountered displaying some kind of discrepancy. Of those cases seven gave a weak and uncertain agglutination with either the horse or the human serum, whereas absorption experiments clearly proved them to be P—. The eighth sample was about a week old and was not agglutinated by any of the sera, in the absorption experiment, however, inhibition of the specific agglutinin to a moderate extent was observed. The ninth sample was agglutinated by the horse serum but not by the human serum and the standard absorption gave a slightly dubious result. On account of scarcity of blood more elaborate absorption experiments could not be performed. Thus in only two cases among the adults a certain diagnosis could not be established, and very likely reexamination of fresh samples might solve the question.

In children the situation is slightly different. Concomitantly with the series of 975 adults 266 children were tested, of which 13 gave dubious results. It has to be taken into consideration, however, that very often the scarcity of blood in samples from children prohibits extensive absorption experiments.

As a last resort in dubious cases the serum of the sample may be examined with regard to the specific anti-P agglutinin which has been found to be present in a majority of the negative bloods.

Receptor strength.

The variation in receptor strength which is one of the outstanding characteristics of factor P has been subject to some investigation by earlier workers. In their original series Landsteiner & Levine found 3 groups according to the intensity of the reactions. Of 265 observations 8.3 per cent were designated ++, 42.3 per cent (+) and 31.3 per cent +— the remaining 18.1 per cent being purely negative. A more exact estimate of the strength has been attained by titration and absorption experiments (Dahr, Andresen, Jonsson), but titrations especially have been hampered by the difficulties in obtaining sufficiently potent sera.

After obtaining a human anti-P serum of considerable strength, provided testtube method and refrigerator temperature is employed, the author has been able to estimate the strength of a great many unselected samples by titration. The results of those titrations are given in figure 1.

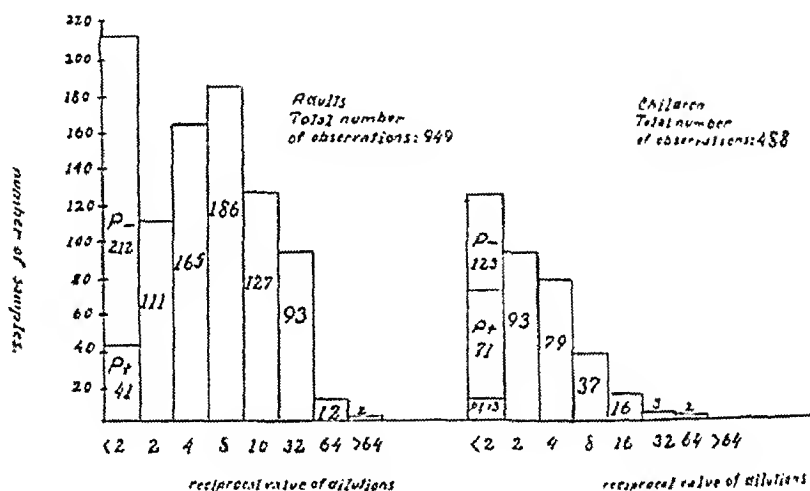


Fig. 1.

Distribution of the receptor strength in adults and children.

As seen from the diagram it is not possible in this way to subdivide the factor in distinct classes according to strength, the graph being approximately of the shape of a normal distribution. By absorption experiments using another human serum a similar conclusion was reached, the absorbing power of the different samples varying gradually from very weak to strong with no sharply defined intervals. Titrations with still another human anti-P serum did not differ in any way from this result.

In children the strength of the receptor is found considerably weaker than in adults. This fact, which is in conformity with the findings within the ABO-bloodgroup system, has been noted by earlier investigators (Andresen, Jonsson), but is contradicted by the results of Dahr (8). It is confirmed in this material by a great

number of titrations with a human serum, the results of which titrations are given in figure 1. It is seen that the peak of the curve in children is at 2 instead of at 8 as in adults, and that a far greater proportion of P+ children does not react at all with this serum.

A number of samples have been examined simultaneously with several different sera by titration and absorption obtaining fairly good, if not complete correlation between the receptor strength independently of the method of examination. As a general rule it is seen that receptors proclaimed weak by examination with one serum are found weak too by examinations with the other available sera and vice versa, no matter whether the strength has been determined by simple titration or by absorption. As an example a diagram of the correlation between the titrations with sera *Leo* and *Gunnar* is shown.

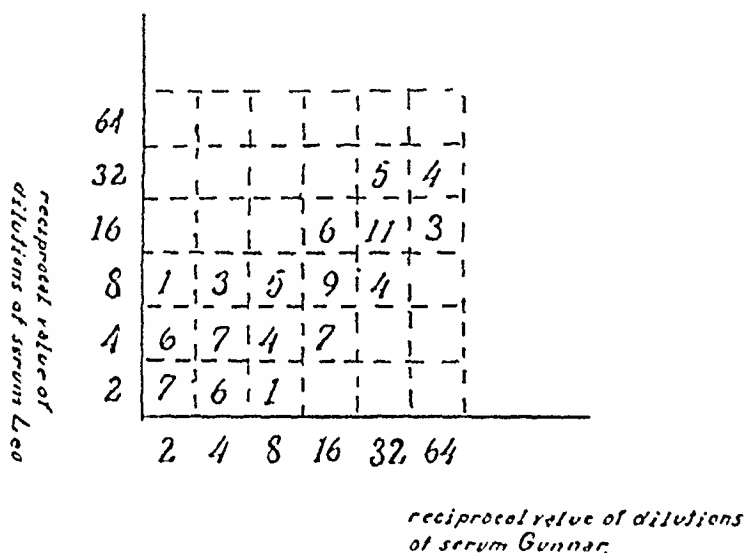


Fig. 2.

89 random blood samples titrated against serum »Gunnar» and »Leo». The final titer of each sample against one of the sera is plotted against the final titer of the same sample against the other serum.

From this general rule a few exceptions have been encountered, but further investigations have not revealed indications of qualitative differences in the P-receptors or the anti-P sera. A number of the minor discrepancies is to be explained by the sera's varying capacity of differentiating the strength. Apparently the homologous sera have a greater differentiating range, the strong P+ having a high titer with these sera, the weakest not reacting at all, whereas the heterologous serum gives more uniform reactions with regard to the final titer.

Attempts at a qualitative differentiation by means of absorption experiments have not been successful. With due regard to the strength of the sera and the receptor strength in the bloods examined, even the weaker receptors by absorption are able to remove all of the specific agglutinin contained in the various sera. On the other hand

it has been established, that the strength is a constant property in adults, and by family investigations it is revealed that this quality depends on homo- and heterozygotism as well as on other hereditary differences.

Anti-P agglutinins of human origin.

Anti-P agglutinins in human sera of sufficient potency for practical purposes are found in a limited number of cases, even if they are considerably more frequent than maintained by Dahr (6). Using a very sensitive technic, however, specific agglutinins are demonstrable in most P-negative sera. As anti-P of human origin is a cold agglutinin the sera to be tested have to be absorbed with regard to the non-specific cold agglutinins, which absorption is done in the refrigerator overnight with half a volume OP— corpuscles. The absorbed sera are tested against 4—5 strong OP+ and 2—3 OP—. In this way 109 P— sera have been tested, 84 being unselected, 25 preferably mothers and children. The results are given in table 5.

Table 5.

	part 1.				part 2.				part 1 + 2	
	men	women	children	total	men	women	children	total	men women children	total
sera containing anti-P	16	23	9	84	2	15	5	25	70	109
sera not containing anti-P	17	7	12		0	1	2		39	

Determinations of specific anti-P agglutinin in sera from P— blood specimens.

It appears from the table that a greater proportion of sera from women than from men and children contains demonstrable agglutinins, which might indicate an immunizing mechanism. In order to, obtain evidence of this hypothesis the mother-child combinations with regard to group P are plotted against the occurrence of anti-P in the serum of mother or child. This does not confirm a theory of a specific immunizing mechanism, provided that the mothers are primiparae. As the bloodsamples originate from cases of disputed paternity this presumption may be justified in a great proportion of cases.

In the second part of table 5 it is seen that specific agglutinins are found in almost 90 per cent, and probably sufficient strong test-bloods might reveal anti-P agglutinins in 100 per cent of P— sera. The author has not had the opportunity of investigating the sera of a series of women who have not been pregnant and consequently the question can not be answered, if the greater percentage of demon-

Table 6.

	serum contain- ing anti-P	serum not containing anti-P
mothers with P+ child	15	4
mothers with P— child	11	2
children with P+ mother	8	5
children with P— mother	6	4

Distribution of anti-P agglutinin in sera of mothers and children tabulated with regard to the P factor.

strable agglutinins in women is due to a nonspecific immunization during pregnancy.

In some instances the author has examined P— sera using albumine suspensions, blocking test and developing test (Coomb's test) (12, 26, 4) without obtaining evidence of incomplete, specific antibodies.

In a number of instances the strength of the agglutinins has been titrated against strong P+ corpuscles as demonstrated in table 7. In a great proportion of cases, however, the amount of serum available was not sufficient for titrating purposes and further 3 of the author's usual test sera of human origin are included in the table. Consequently this may not represent the real proportions of the strength of the agglutinins, but at any rate it depicts the range of the variation.

Table 7.

	< 2	2	4	8	16	32	64	128	reciprocal value of dilutions.
men	2	1	1	2	1	0	2	0	
women	3	7	4	0	3	0	2	0	
children	1	1	2	1					
total	6	9	7	3	4	0	4	0	

Final titer of anti-P agglutinins in 33 human sera when tested against strong P+ corpuscles.

Some of the most potent anti-P sera which the author has encountered, originate from men. On interrogation of these men, no information was obtained of injections or transfusions which might have acted as immunizing agents. The agglutinin may thus be considered as a natural iso-agglutinin of greatly varying strength, in most cases, however, being very weak.

In view of the maintained character of anti-P of human origin as a cold agglutinin, some experiments have been made with the purpose of establishing the optimum temperature of the agglutination. Two human and one horse serum were tested against strong P+ corpuscles

incubating 2 hours by various temperatures. After centrifugation — 2000 rev./per min. for 1 minute — the reactions were read macroscopically by aid of a hand lens after gentle shaking. The results are given in figure 3 from which is seen that the human sera react considerably stronger at 6 degrees centigrade than at 20, and do not react at all at 30 or 37 degrees centigrade. The reactions of the horse serum are also weaker at high temperatures hut to a lesser degree than the human sera and there is no appreciable difference between the reactions at 6 and 20 degrees centigrade.

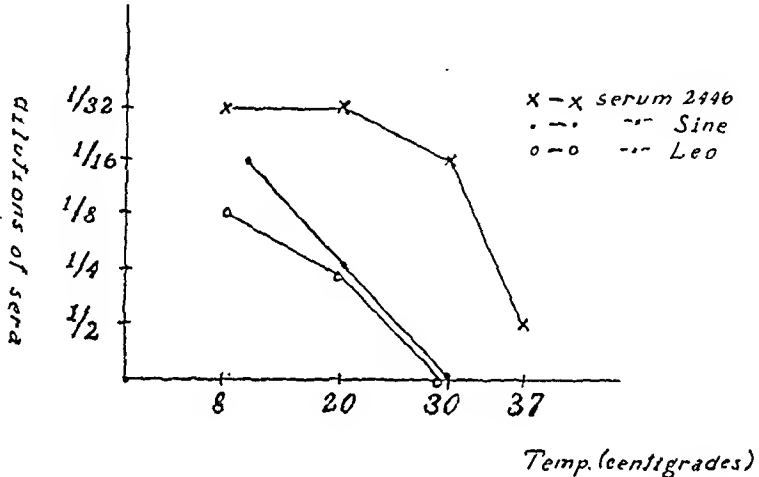


Fig. 3.

Titration of a horse serum (2446) and two human sera against strong P+ corpuscles at different temperatures.

The author has not been able to produce agglutination with human sera at body temperature, although such has been observed using albumin suspension of the corpuscles (Freisleben: personal communication). On the other hand the fact that agglutination in vitro is difficult to produce does not exclude the possibility of agglutination in vivo.

The significance of factor P.

The significance of factor P in clinical medicine is probably very slight. The possibility of a P-positive foetus immunizing as P-negative mother and in consequence contracting erythroblastosis can not be excluded, but instances of erythroblastosis of this genesis have not been published, and as mentioned above, this investigation has not confirmed the hypothesis of a specific immunizing agent as a cause of anti-P antibodies.

Following bloodtransfusions haemolytic reactions caused by anti-P antibodies have been encountered, but probably they are exceedingly rare. Wiener & Peters (25) have demonstrated an anti-P agglutinin in a bloodspecimen from a patient who had a haemolytic reaction following a blood transfusion. Wiener & Unger (27) report a case of an anti-P agglutinin presumably caused by immunization and found in

the blood of a patient in the course of a series of blood transfusions.*)

The chief value of P-grouping, however, is in forensic medicine in cases of disputed paternity. In a number of cases in Germany and Sweden (Dahr, 9, Wolff, 28, Jonsson, 14) P-grouping has been used to prove non-paternity, the evidence, however, being regarded as inferior to the evidence of ABO- and MN-grouping. In Denmark P-grouping as yet is only used for legal purposes in selected cases preferably coupled with a general comparative anthropological examination, and at that it is considered only circumstantial evidence.

The medico-legal aspect of P-grouping will be discussed in some detail in connection with an investigation on the heredity of factor P which will be published in a later communication.

Summary.

After a short survey of the previous communications on factor P the results of the author's investigations are presented.

1. The technique of the investigation is described, the importance is stressed of using animal as well as human sera, and further the necessity of absorption experiments in determining weak P receptors is mentioned.

2. In determining an unselected sector of the Danish population the frequency of factor P in adults was found to be 78.9 per cent in 2345 observations. In children the frequency was somewhat lower indicating that the receptor is not fully developed at birth. In analysing the material the reliability of the determinations is discussed.

3. An appraisal of the variation of the receptor strength is attained by titrations with a human anti-P serum. The results are shown in a graph representing approximately a normal variation curve and it is concluded, that a subdivision of the P factor in distinct groups according to strength is not possible. On the other hand it is postulated that the receptor strength is a constant property depending on homo- and heterozygosity as well as on other hereditary differences. Investigations on the heredity of factor P justifying this postulate are going to be published in a later communication.

4. In examining a number of sera from P— blood specimens anti-P agglutinins have been demonstrated in a majority of cases, most frequently in women, but also in men and children. It has not been possible to find evidence of any specific immunizing process. In comparing anti-P sera derived from different sources approximately identical results have been obtained, and no evidence of qualitative differ-

*) P. Moureau (Rev. Belge Sciences Med. tome XVI: 258. 1945) has reported a fatal case of hæmolytic reaction following blood transfusion due to anti-P antibodies

ences between anti-P agglutinins or between P factors of different strength has been found.

The clinical and forensic significance of P grouping is briefly mentioned.

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ADDENDUM:

Since this paper was submitted for publication *R. Sanger, S. D. Lawler* and *R. R. Race* (Revue d'Hématologie, tome 4, no. 1. 1949. pag. 28) have reported on an investigation of factor P. The observed frequency of P+ in 500 observations was 74 per cent. In comparing these results with those of the author's χ^2 is found to be about 5.7 for one degree of freedom. Consequently the difference may well be significant and may be explained by a lower frequency of the gene for P in London than in Denmark. On the other hand the possibility cannot be excluded that the different results obtained by various investigators are due to differences of the technic employed.

INFLUENCE OF SODIUM CHLORIDE ON GROWTH OF STAPHYLOCOCCI AND SOME OTHER BACTERIA

By Astrid Fagraeus.

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The isolation of staphylococci from clinical material (throat swabs, pus and feces) is complicated by the presence of other organisms which grow more vigorously on the usual media than do staphylococci. However, Koeh observed that staphylococci grew out in pure culture from bacterial mixtures (staphylococci, *B. coli*, *Salmonella*, streptococci and the diphtheria bacillus) if the mixture were inoculated in broth containing 15—20 % sodium chloride. Chapman made use of this observation in preparing a media containing 7.5 % NaCl for the isolation of staphylococci from mixtures of bacteria and this media (referred to as phenol mannitol plate) has been used in our laboratory for some time. It has not proved entirely satisfactory because other bacteria, particularly *B. proteus* and some *B. subtilis* strains are not always sufficiently inhibited at this concentration; furthermore, even the staphylococci are somewhat inhibited at this concentration. Accordingly, attempts were made first to cultivate fecal samples in serum broth containing 6.5 per cent sodium chloride, followed by a subculture on phenol mannitol plates. This procedure was found to yield considerably more staphylococci than did a smear on the Chapman plate alone. The same applied to the isolation of staphylococci from the nose and pharynx. However, these results will be treated in more detail in another connection.

At these preliminary examinations a broth containing 6.5 per cent sodium chloride was used, i. e. slightly less than the quantity included in the Chapman substrate. Nevertheless, an increase in the content of sodium chloride might give a still greater accumulation of staphylococci at cultivation of a mixed infected material. The present investigation was undertaken in an attempt to define the concentration of sodium chloride which maximally inhibits other organisms while permitting an adequate growth of staphylococci.

Materials and methods:

Bacteria. 56 coagulase-positive strains of the *Staphylococcus aureus* were tested. All had been isolated from fecal samples. 15 *B. coli* strains and 10 *Proteus* strains had the same origin. One of the 5 *Subtilis* strains was obtained from feces.

The growth of these different bacteria in varying sodium chloride medium was examined, separately as well as in different combinations.

Media: The Chapman media as used in this experiment contained 7.5 % sodium chloride, 0.03 % phenol and 1.0 % mannitol. Ordinary blood plates were also used for comparison. Broth was prepared containing 15 % horse serum and the following percentages of sodium chloride: 0.5, 6.5, 7, 8, 10, 12, 14, 16 and 18.

Methods. For *unmixed* cultures, 0.1 ml. of a 20 hour broth culture (diluted to 10^{-6} for staphylococci, and to 10^{-2} for the other bacteria) was inoculated into a series of tubes containing 6 ml. of broth of varying sodium chloride concentration. Small inocula were used so that the resulting sparse growth could be better estimated by visual examination. The amount of the inoculum was usually determined by plating the same sample on agar and reading after 24 hours incubation. The test series were incubated at 37° for 18–20 hours and the growth determined directly on a scale from 0 to +++. This was sometimes checked by plate counting (from a subculture on agar).

For *mixed* cultures 0.1 ml. of a 20 hour culture (diluted as above) was inoculated in the same way, but at the same time a similar quantity was plated on an ordinary blood agar and on a phenol mannitol plate. After incubation for 18–20 hours at 37°, 0.1 ml. of the test broth cultures was subcultured on the same substrates. In a few instances a smear was also made from the broth cultures diluted to 10^{-3} . The blood plates were studied after about 24 hours, the phenol mannitol plates a second time after incubation for a further 24–48 hours.

Results:

A. Examination of the sodium chloride tolerance of various bacteria.

1. *Staphylococcus aureus* (Table 1).

After cultivation of very small quantities of bacteria (0.3–12 bacteria per ml. of broth) for 24 hours at 37° C a fairly good growth was observed in the broth tubes containing up to 8–10 per cent sodium chloride. All of 56 examined strains grew distinctly, at macroscopic examination, in broth containing 10 per cent sodium chloride. After this a slight inhibition set in and, at 14 per cent, growth was obvious in only 22 of 32 cases. On visual inspection of the broth tubes containing 16–18 per cent sodium chloride, no growth was discernible. The bacterial count in the plates, however, disclosed slightly more than the number of inoculated bacteria in these broth tubes. Such a plate count was performed in 22 cases, as a rule confirming the observations made at direct observation. In broths containing 6.5–7 per cent sodium chloride, the bacterial growth was fairly rich and was generally not affected to any noteworthy extent by an increase in the content of sodium chloride to 8–10 per cent. After this a gradual inhibition took place, and after 14 per cent (i. e. in broths containing 16–18 per cent) the growth was markedly reduced.

Even visual inspection had shown that broth tubes with the usual

Table 1.
Growth of 22 strains of *Staphylococcus aureus* in broth with varying concentrations of sodium chloride. See the text for methods.

Strain nr.	Inoculum Number of bac- teria inoculated pr. ml broth	Growth										
		Millions of bacteria pr ml broth containing sodium chloride in concentrations										
		0.5 %	6.5 %	7 %	8 %	10 %	12 %	14 %	16 %	18 %		
68	2.5	6.0	25	29	38	8.3	3.1	2.8	0.06	0.002		
69	3.1	18	25	23	21	8.7	3.5	1.0	0.04	0.002		
100	1.7	1.5	22	19	16	21	5	3.4	0.07	0.003		
6922	5.1	3.4	53	65	27	14	14	2.9	—	—		
6931	4.7	20	71	50	33	26	8.0	1.5	—	—		
6937	0.5	0.4	5.9	7.5	2.0	1.3	0.7	0.0005	—	—		
6938	0.3	1.5	22	6.5	8.0	3.8	0.0005	0.002	—	—		
6944	12	14	32	43	26	24	31	0.8	—	—		
6946	1.0	9.8	14	14	10	7.6	0.3	0.002	—	—		
8045	1.0	0.1	4.3	1.9	0.5	0.7	0.008	—	—	—		
8158	5.0	14	16	24	19	13	3.4	2.3	0.04	0.0005		
8252	1.0	0.9	12	5.2	16	2.2	0.03	0.003	0.0008	<0.0001		
8269	2.0	5.5	8.5	9.4	21	5.0	2.3	1.8	0.02	0.00008		
8280	7.1	35	21	60	17	21	3.8	1.9	0.01	0.00009		
8297	6.5	16	55	30	23	11	1.3	2.0	0.2	0.004		
8788	5.0	17	13	8.4	—	1.2	0.2	3.0	0.2	0.0006		
9070	3.0	4.0	28	17	22	2.9	1.5	1.9	0.3	0.001		
9221	2.5	4.5	9.6	20	6.4	2.7	1.0	1.0	0.1	0.002		
9705	2.7	6.8	17	7.2	5.6	2.5	1.0	0.8	0.05	0.00009		
9740	2.3	20	20	17	7.4	5.0	3.2	0.7	0.04	0.0008		
9743	0.7	11	18	18	18	5.4	—	1.3	0.03	<0.00001		
9819	1.8	5.5	12	11	10	6.6	3.7	2.1	0.05	0.008		

Table 2.

Growth of 5 strains of *B. coli*, isolated from feces, in broth with varying concentrations of sodium chloride.

Strain nr.	Inoculum per ml broth Number of bact. inoculated	Growth Millions of <i>B. coli</i> in broth containing sodium chloride in concentrations					
		0.5 ‰	6.5 ‰	7 ‰	8 ‰	10 ‰	12 ‰
1	170,000	180	3.0	6.6	0.07	0.01	0.01
2	340,000	55	6.5	11	0.15	<0.01	<0.01
3	240,000	160	13	10	0.3	<0.01	<0.01
4	240,000	23	4.0	0.2	0.01	0.02	<0.01
5	220,000	50	8.5	4.0	4.0	<0.01	<0.01

mixture of sodium chloride (0.5 per cent) often had less sediment of bacteria, or less turbidity, than broth with 6.5—8 per cent (10 per cent). Moreover, the plate count was higher at the latter content of sodium chloride. In only 2 of the 22 counted strains the total number of bacteria was greater in the broth with 0.5 per cent of this compound. The counts were performed on different occasions and 2 (partly 3) different series of broth were tested.

2. Other bacteria (Tables 2 and 3).

The 15 *coli* strains were distinctly inhibited even at a content of sodium chloride of only 6.5 per cent. 10 strains disclosed pronounced inhibition of growth at an admixture of 10 per cent of sodium chloride. Also in the other cases, there was a conspicuous difference in bacterial density between a broth with 8 and one with 10 per cent sodium chloride.

The inhibition of growth of *Proteus vulgaris* was generally insignificant at 6.5 per cent sodium chloride. They often grew excellently even at 8 per cent. At 10 grams %, however, there was a distinct growth inhibition.

Table 3.

Growth of 6 *Proteus* strains, isolated from feces, in broth with varying concentrations of sodium chloride.

Strain nr.	Inoculum per ml broth Number of bact. inoculated	Growth Millions of bacteria in broth containing sodium chloride in concentrations						
		0.5 ‰	6.5 ‰	7 ‰	8 ‰	10 ‰	12 ‰	14 ‰
P 17	53,000	11	7.5	8.2	9.4	0.01	0.02	<0.00001
P 18	74,000	11	14	7.7	3.4	0.006	0.04	0.0001
P 19	25,000	11	3.4	3.5	0.8	0.003	<0.001	—
P 20	40,000	33	11	6.0	7.8	0.03	0.001	—
P 21	40,000	13	15	7.0	12	0.3	0.003	—
P 22	22,000	39	23	2.2	6.5	0.8	0.01	—

The 5 *Subtilis* strains grew abundantly even at 10 per cent and 4 of them even at 12 per cent. The growth in broth with these concentrations of sodium chloride was, nevertheless, not so rich as in ordinary serum broth.

B. Mixed cultures:

1. *Staphylococcus aureus* and *B. coli* (Table 4).

In serum broth inoculated with staphylococci and coli at proportions of 1:100,000, *B. coli* were present in almost pure culture after 20 hours. When the broth cultures were inoculated on phenol mannitol plates, a moderate or sparse growth of staphylococci was obtained but, with a further dilution of 1:1000, there was no growth on phenol mannitol plates. This proves that the quantity of staphylococci was insignificant. On blood plates, practically only coli grew. On the other hand, on phenol mannitol and blood plates to which broth cultures containing sodium chloride were transferred the growth of the staphylococci was greater in proportion as the amount of sodium chloride in the broth was increased. From 10 per cent sodium chloride broth, a blood plate disclosed a sparse growth of *B. coli* and, at 12 per cent, pure cultures of staphylococci grew in several instances, especially when the broth cultures were diluted 1000 times. When the bacterial cultures used for inoculating the broth tubes were grown on blood plates only *B. coli* grew and no growth at all was noted on phenol mannitol plates.

2. *Staphylococci* and *Proteus vulgaris* (Table 5).

When a 20-hour-broth culture of staphylococci as well as *Proteus* was smeared on phenol mannitol plate, a moderate growth of staphylococci would appear after 48—72 hours. When the broth was diluted 100 times, the result was the same as when staphylococci and *B. coli* were cultivated together, i. e. negative. When, on the other hand, broth containing 6.5 per cent or more of sodium chloride was cultivated on phenol mannitol plates, the staphylococci grew in moderate or abundant quantities even when the broth was diluted 1000 times, and cultures of undiluted broth gave a very rich growth of staphylococci. On blood plates, *Proteus* completely dominated in cultures from ordinary broth and those containing 6.5—8 per cent sodium chloride. It may be seen from Table 3, that 10 per cent clearly inhibited growth of *Proteus*. Further, from mixed cultures of staphylococci and *Proteus* on broth containing 10—14 per cent sodium chloride, a marked growth of staphylococci was obtained on blood plate, and even diluted broth yielded pure cultures of *Staphylococcus aureus*.

3. *Staphylococci* and *Subtilis* (Table 6).

The *Subtilis* group of bacteria showed a high resistance to sodium chloride and grew without difficulty even at a concentration of 10—12 per cent. Due to the tendency of *B. subtilis* to conglomeration and the formation of a pellicle on growth in broth, no quantitative determina-

Table 4.

After cultivation of about 200,000 coli bacteria and 2 (0.3—3.7) staphylococci on broth with the above-mentioned sodium chloride, the broth was incubated for 20 hours at 37° C. Then 0.1 ml. of undiluted broth or broth diluted to 10—3 was cultivated on blood plates and phenol mannitol plates. The bacterial growth was determined after 48 hours and recorded as abundant (+++), fairly abundant (++), moderate (+), sparse ((+)) and non-existent (0).

Nr.	Broth di- luted	Broth containing sodium chloride in concentrations											
		0.5 %				6.5 %				8 %			
		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate	
		Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.
Coli 1	1/1	+	(+)	+	+	+	(+)	0	+	+	+	+	+
+													
8026	1/1000	0	0	+	+	0	0	0	+	+	+	+	+
Coli 3	1/1	0	(+)	+	+	+	0	0	+	+	+	+	+
+													
8157	1/1000	0	0	+	+	(+)	0	0	+	+	+	+	+
Coli 4	1/1	0	+	+	+	+	(+)	0	+	+	+	+	+
+													
8158	1/1000	0	0	+	+	+	(+)	0	+	+	+	+	+
Coli 5	1/1	0	+	+	+	+	0	0	+	+	+	+	+
+													
9221	1/1000	0	0	+	+	+	0	0	+	+	+	+	+
Coli 6	1/1	(+)	+	+	+	+	0	(+)	+	+	+	+	+
+													
8269	1/1000	0	0	+	+	0	(+)	0	+	(+)	+	+	+

Table 5.

Growth of *Staphylococcus aureus* in broths, with varying content of sodium chloride, inoculated together with about 20,000 times more *Proteus*. As regards the procedure, see the text and also Table 4.

Nr.	Broth di- luted	Broth containing sodium chloride in concentrations											
		0.5 %			6.5 %			8 %			10 %		
		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate	
		Prot.	Staph.	Prot.	Staph.	Prot.	Staph.	Prot.	Staph.	Prot.	Staph.	Prot.	Staph.
P 3	1/1	+	++	++	0	(+)	++	++	++	0	++	++	++
+													
9070	1/1000	0	+	++	0	0	++	++	(+)	0	++	++	++
P 15	1/1	+	++	++	0	+	++	++	++	0	++	++	++
+													
9221	1/1000	0	0	++	0	0	++	++	++	0	++	++	++
P 16	1/1	+	++	++	0	(+)	++	++	++	0	++	++	++
+													
9705	1/1000	0	0	0	0	0	++	++	++	0	++	++	++
P 17	1/1	+	++	++	0	+	++	++	++	(+)	++	++	++
+													
9740	1/1000	0	0	++	0	0	++	++	++	0	++	++	++
P 18	1/1	(+)	++	++	0	+	++	++	++	0	++	++	++
+													
9743	1/1000	0	++	++	0	0	++	++	++	0	++	++	++

Table 6.

Simultaneous culture of *B. subtilis* and *Staphylococcus aureus* in broths with varying sodium chloride content (cp. the text).

Nr.	Broth di- luted	Broth containing sodium chloride in concentrations																	
		0.5 ‰						8 ‰						10 ‰					
		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate		Phenol mannitol		Blood plate		Phenol mannitol	
		Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.	Subt.	Staph.
S 1 +	1/1	+++	0	+++	(+)	+++	+	+++	+	+++	+	+++	+	++	+	++	+	+	+
8026	1/1000	0	0	+++	0	++	0	+++	++	0	0	+++	++	0	0	+++	0	0	+
S 3 +	1/1	++	(+)	+++	0	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+
8157	1/1000	0	0	+++	0	++	0	+++	++	0	0	+++	++	0	(+)	+++	0	(+)	+
S 4 +	1/1	0	+	+++	(+)	+++	+	+++	+	+++	+	+++	+	0	+	++	+	+++	+
8236	1/1000	0	0	+++	(+)	++	0	+++	+	0	0	+++	+	0	0	+++	0	(+)	+
S 5 +	1/1	+++	0	+++	0	+++	+	+++	+	+++	+	+++	+	+	+	+++	+	+++	+
8269	1/1000	0	0	+++	0	++	0	+++	++	0	0	+++	++	+	0	+++	0	0	(+)

tion could be made according to the plate method. However, the fact that blood plates from broth cultures with staphylococci and *B. subtilis* contained an increasing number of staphylococci, in proportion as the content of sodium chloride rose to 12 per cent, proved that even the growth of *B. subtilis* bacteria is inhibited by larger concentrations of sodium chloride.

C. Growth of Staphylococcus aureus on blood plate and phenol mannitol plate.

It was mentioned in the introduction that the phenol mannitol substrate seemed to have an inhibiting effect also on the growth of staphylococci. A study of Tables 4 and 6 will show that the staphylococci, in several cases, were more numerous on blood plates than on phenol mannitol plates when inoculated with the same amount of broth culture containing a fairly large quantity of sodium chloride. In order to examine more closely whether any difference existed in the growth on the different substrates, a cultivation was made of an identical quantity of staphylococci on a blood plate and on a phenol mannitol plate, the number of colonies being counted after incubation for 24 and 48 hours at 37° C. The colony count was the same on the blood plates after 24 and 48 hours. On the phenol mannitol plates, the quantity was often doubled at the second registration and the colonies also became considerably enlarged. A comparison between the number of colonies in the various substrates revealed that the total number after 48 hours was the same, or but slightly greater, on blood plate in 6 out of 16 cases. In 8 cases, 50—100 per cent more colonies grew on the blood plate, the number on the phenol mannitol plate predominating in only 2 cases.

Discussion.

The purpose of the present investigation was, in the first place, to determine the amount of sodium chloride in broth that, at cultivation of mixed- infected material (particularly feces) gives the greatest accumulation of *Staphylococcus aureus*. It is necessary that such an accumulative substrate should strongly inhibit growth of other bacteria in the sample while not restraining the growth of staphylococci to any marked extent. At cultures of fecal specimens, the *Coli* and *Proteus* group of bacteria, in particular, grow more abundantly than staphylococci. Also *B. subtilis* may effectively mask the existence of staphylococci in feces, as in pharyngeal specimens.

As regards the *B. coli* and *Proteus*, a concentration of sodium chloride of 10 per cent in a broth is sufficient for marked inhibition of the growth of these bacteria. At 12—14 per cent the inhibition becomes still more pronounced. On the other hand, the *Staphylococcus aureus* still grows abundantly at a sodium chloride quantity of 10 per

cent, even though a certain inhibition may sometimes be observed on comparison with the number of bacteria in broth containing 6.5—7 per cent of this compound. A sodium chloride concentration of 12—14 per cent causes a more distinct inhibition of growth. Only 16—18 Gm. of sodium chloride per 100 ml of broth will, however, result in a marked reduction in the growth of the staphylococci.

It is noteworthy that after incubation for 20 hours, often more staphylococci (sometimes considerably more) are observed in broths with 6.5—7 per cent sodium chloride than in ordinary serum broths which contain only 0.5 per cent salt. As this was demonstrated in 3 different series of broth substrates, it can hardly have been coincidental. Thus, it seems as though a moderate supply of sodium chloride to a substrate would activate growth of staphylococci. *Chapman* apparently made the same observation, maintaining that staphylococci coagulate plasma better when growing on 7.5 per cent proteus lactos agar. It has been proved, provided a staphylococcus produces coagulase at all that the capacity of the broth culture to coagulate plasma is proportional to the growth of the bacteria. Thus, when a series of broth cultures is tested, containing from 0.5 to 14 per cent sodium chloride, the coagulase-reaction may be negative in the lowest concentration and positive in 6.5 per cent broth if the growth in the latter medium is much more marked than in the former. Broth cultures containing 14 per cent sodium chloride may give a positive reaction with a heavy bacterial growth, if the inoculation is sufficiently large.

In choosing the concentration of sodium chloride which is most suitable for isolating *Staphylococcus aureus* from a mixed flora attention must be paid, partly, to the sodium chloride sensitivity of this bacterium and, partly, to the bacteria whose growth is to be inhibited. If, as in the present investigation, a more effective inhibition of the growth of *B. coli*, *Proteus* or the *Subtilis* group of bacteria is needed, at least 10 Gm. per 100 ml of broth of sodium chloride must be used. In this medium, staphylococci still grow abundantly. When the quantity of sodium chloride is further increased, this may inhibit growth of *Proteus* slightly more than before, for example, but it will also reduce the growth of the staphylococci. At any rate, 14 per cent sodium chloride represents from a practical point of view, the maximal sodium chloride concentration. The advantage of this high content of sodium chloride in broth, viz, the possibility of obtaining pure cultures of staphylococci from mixed cultures on blood plate, will scarcely equal the disadvantage that certain strains will not tolerate such a large quantity of sodium chloride. In addition, an increase of the concentration above 10 per cent would seem to be unnecessary, because even at this percentage the staphylococci will grow distinctly if cultivated on a blood plate, together with *Proteus*.

Although the use of this concentrations of sodium chloride does

not inhibit the growth of *B. subtilis* to such an extent as it does the other organism in these experiments its growth is sufficiently suppressed to allow good multiplication of the staphylococci.

Summary.

1. All the 56 coagulase-positive strains of the *Staphylococcus aureus* disclosed abundant growth in serum broth containing up to 8—10 per cent sodium chloride. A marked inhibition of growth occurred at a sodium chloride concentration of 16—18 per cent.

2. *B. coli* and *Proteus vulgaris* were clearly inhibited by a sodium chloride content of 10 per cent. The growth of *Proteus* was further restrained when the concentration of sodium chloride was increased to 14 per cent.

3. Cultivation of the *Staphylococcus aureus* together with *B. coli* and *Proteus* on a 10—14 per cent sodium chloride broth caused a good accumulation of the staphylococci.

4. A 10 per cent sodium chloride broth was considered to be the best accumulative substrate for staphylococci.

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DECALCIFICATION INVESTIGATION WITH CONTRIBUTIONS TO THE HISTOLOGICAL TECHNIQUE OF THE INTERNAL EAR

By *Harald Kristensen.*

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After some years in experimental histological research on the internal ear of guinea-pigs it has become clear that the histologic technique of this special field is somewhat deficient, owing partly to the situation of the internal ear, enclosed in bone, and partly to the extreme sensitivity of the internal ear to outer influences.

As late as 1940 this deficiency was pointed out by *Werner* in his comprehensive book about the labyrinth. In this connection, *Werner*, also wrote that a closer study of fixation, decalcification and embedding was necessary. In other fields *Lillie* emphasized that quick and satisfactory staining of the bone marrow still offered a technical problem.

In most histologic manuals several decalcification methods are summed up, but only very few and inextensive studies have been performed to find out what the principle of decalcification is and which method is preferable.

Schaffer (1902) tried to bring order from the confusion by means of a systematic testing of the then known decalcification methods. He compared decalcification time to swelling or shrinking of tendon and bone tissues. After this, the author recommended decalcification in 5% aqueous solution of nitric acid — a method which is still the most widely used.

The first progress in decalcification methods since the work of *Schaffer* was achieved by *Evans & Krajian* (1930), when they published a new method of decalcification. The decalcification agent was composed of equal parts aqueous 85% formic acid and 20% aqueous sodium citrate. In comparison with the effect of nitric acid, the authors found that the cellular elements were practically unaffected and staining was perfect. No mention was made of the decalcification time.

In regard to decalcification of the bone marrow, *Lillie* (1944) published a comprehensive study on various decalcification agents and their relation to the Romanowsky staining. He was of opinion that a control of the injury to bone marrow staining would be possible through control of the initial pH. He therefore made a series of buffered mixtures of sulfurous acid and formic acid for comparison with nitric acid, trichloroacetic acid and acetic acid. 5% aqueous solution of formic acid was found the most satisfactory.

The problem of decalcification is that tissues containing bone cannot commonly be used for sections and the method of grinding sections is excluded for cytologic purposes. In some way one must alter the consistency of the tissues, in general soften. This only can be done by extraction of the inorganic bone components. This again is to say that the bone-containing tissue must be submitted to an acid action inasmuch enzymatic procedures can be excluded in cytologic researches. But this acid action also influences the organic tissue components and alter their stainability.

During some studies over the influence of noise upon the internal ear (spiral ganglion and spiral organ) I tried to stain by means of *Einarsans* galloeyanin. This was for obvious reasons important because: 1) Galloeyanin stains the Nissl-substance elective, and 2) Galloeyanin stains progressively, excluding any mistakes due to differentiation. The same points of view I have found expressed in »Galloeyanin staining as a specific method for producing protein-inclusions in liver cells« by *Lagerstedt*.

I did not succeed staining by means of this method partly because my preparations were embedded in celloidin, partly because decalcification had taken place in 5 % aqueous nitric acid.

In order to try to find a method of decalcification unusually gently in action, without too much reduction of decalcification power, a systematic, comparative investigation was undertaken of a number of known decalcification agents. The investigations were made as model-experiments using pieces of kidney, liver, brain and spinal cord as control for stainability, and small pieces of human rib as control of the decalcification. The employed acids were lactic acid, formic acid, citric acid, acetic acid, trichloroacetic acid, phosphoric acid, chromic acid, sulfurous acid, sulfuric acid, nitric acid, hydrochloric acid and various mixtures. After the decalcification the tissues were embedded, cut and stained by means of various sorts of hematoxylin and by means of galloeyanin. Now the preparations were examined in comparison-microscope. The result was that one had to find the best decalcification fluid among the organic acids.

Meanwhile the aforementioned experiments were not satisfactory on account of highly varying results in spite of same procedure. Therefore, in order to obtain more reliable results in further decalcification experiments I made some bone platelets as decalcification test objects and some nucleus preparations as test objects for the stainability after the decalcification.

The decalcification test objects were made from fresh *substantia compacta* (from the fore-leg of a horse). They were made like platelets, 2 mm thick, with a diameter of 7.5 mm and a weight of 170 mg. The test objects for stainability were made from extracted nuclei of spleen (from calf). The nucleus mass was centrifuged to a solid block which was afterwards fixed in 70 % alcohol and embedded in paraf-

fin. Sections of 20 micron were cut and mounted on slides. These sections (after removal of paraffin) together with the bone platelets were put into the aforementioned acids (of various normality and temperature).

The rate of decalcification and the decalcification time was controlled by means of X-ray photographs. At the end of the decalcification the nucleus preparations were treated as ordinary sections and stained by means of gallocyanin and now it was possible to express the intensity of staining in figures by means of a Pulfrich photometer.

In all trials it was found that 1 N and 2 N formic acid gave relatively the best nuclear staining at a not too long decalcification time.

Following investigations on formic acid of various strength proved that 4 N formic acid, of pH 1.1, gave the fastest decalcification but with a somewhat impaired nuclear staining, compared to formic acid of lower normality. Formic acid of higher normality showed a greater injuring action and at the same time the decalcification proceeded more slowly, especially in the case of concentrated formic acid, which decalcified so slowly that the tissue was destroyed.

As by another trial a certain parallism was ascertained between the pH of the decalcifying agent on one hand and decalcifying time and nuclear staining on the other, so that a low pH gave quick decalcification but inferior nuclear staining and vice versa. Furthermore one did not like to use a pH lower than for the common fixatives. It therefore was quite pertinent to try to preserve the decalcifying effect of formic acid with simultaneous diminution of the actual hydrogen ion concentration. I therefore tried a buffer solution of formic acid and the easily soluble sodium salt of this acid.

Even the first trials succeeded surprisingly well and new investigations were started with formic acid of various normalities mixed with 1 N sodium formate in various proportions, in order to find a mixture with optimal decalcifying effect and maximal pH.

Of the various compositions, mixture No. 1 (equal parts of 1 N formic acid and 1 N sodium formate, pH 3.8) decalcified in 5 days, mixture No. 5 (equal parts of 2 N formic acid and 1 N sodium formate, pH 3.25) decalcified in 60 hours, mixture No. 8 (equal parts 4 N formic acid and 1 N sodium formate, pH 2.7) decalcified in 36 hours while mixture No. 10 (equal parts of 8 N formic acid and 1 N sodium formate, pH 2.2) decalcified in scarcely 24 hours.

By photometer examinations on preparations stained with gallocyanin, lowered nuclear stainability was found in those which had been in mixtures with lower pH levels. However the difference was not great; and therefore the fastest decalcifying mixture (No. 10) was preferred.

It is noticeable that the pH of this mixture of formic acid and sodium formate is the same as $\frac{1}{10}$ N formic acid which, however, takes

about 5 days for complete decaleification; and what is more strange, it decaleifies faster than 1 N formic acid with pH 1.75. Furthermore it decaleifies somewhat faster and much better than $\frac{1}{10}$ N nitric acid or hydrochloric acid with pH 1.0 and, moreover it is apparently superior to the mixture of formic acid and sodium citrate with pH 1.75.

Overdecaleification, by using twice the time necessary, shows a somewhat lowered nuclear stainability photometrically, but less so than other known decaleification agents under similar circumstances.

The treatment did not alter the color or the appearance of the bone blocks. They were found uniformly decaleified and easy to cut, even after embedding in paraffin. This is important for the problem under study, because embedding the labyrinths in paraffin is a necessary condition in carrying out the above mentioned staining method.

The only after-treatment used was washing in running water for 24 hours. The preceding treatment with sodium sulfate was omitted because a volumetric, pyenometric investigation showed no advantage from use of sodium sulfate, but rather sooner a distinct, though slight lowering of the stainability.

Now it was quite natural to try to transfer this decaleification and embedding methods to the histologic procedure of guinea-pig labyrinths.

The decaleification rate was very uniform. It lasted about 48 hours, twice the time necessary for nitric acid to decaleify.

As xylene before the paraffin embedding hardened the decaleified bone pretty much I tried to avoid this intermedium, taking instead the tissue blocks directly from water through dioxan to paraffin. Meanwhile this procedure made the tissues shrink rather much.

The following procedure was found the most fit:

Dehydration in alcohol (70 % — 96 % — 100 %) — cedar oil 12 hours ligroin 2 hours — and paraffin.

The employed paraffin contained about 20 % yellow bee wax. This high wax contents kept the paraffin easy to cut in spite of cooling the tissue blocks before the cutting. In this manner I was able to cut sections 6—7 microns thick.

In these sections the Nissl-granules of the spiral ganglion stained excellently by means of galloeyanin. According to *Einarson* this means that the formic acid-sodium formate decaleification method as opposed to other known decaleification methods preserves the nucleoproteins of the ganglion cells.

Summary.

Following several experimental investigations, an improved method of decaleification has been devised. The principle of this method is to obtain complete decaleification without diminishing the stainability of the Nissl-granules by means of galloeyanin. This is accomplished by the help of a buffer solution of equal parts of 8 N formic acid and

1 N sodium formate (pH 2.2). After-treatment only consists in rinsing in flowing water for 24 hours. Dehydration is in alcohol (70 %, 96 %, 100 %) — cedar oil-ligroin. Embedding in paraffin follows. By means of this procedure I succeeded in staining the Nissl-substance of the internal ear ganglia with gallocyanin.

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THE X STRAIN OF *SH. PARADYSENTERIAE* (FLEXNER) — APPARENTLY A VALID TYPE

By Rolf Saxholm.

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As a result of the investigations of *Boyd* (1938, 1940) and *Wheeler* (1944 a, 1944 b) on *Sh. paradysenteriae* (Flexner), the types X and Y are now often regarded as degenerated laboratory strains without type-specific antigens. Recently *Kauffmann* and *Ferguson* (1948) concurred with this view. *Wilson* and *Miles* (1947) have suggested that the numbering of the Flexner types should be delayed till the position of X is cleared up. *Weil et al.* (1944) maintain that X and Y have type-specific antigens, but said of X that: »We have found this type up to the present time only in stock strains.« Further on they said: »Whether antigen VII (X) occurs as a sole primary antigen in freshly isolated strains, or whether it comes only to the fore in old culture strains after loss of other somatic antigens — as contended by *Boyd* — is a question that only future experience can settle.« At a later date (1947), *Weil* in a preliminary report stated that he had identified a recently isolated strain as X. *Elrod* and *Wormus* (1946) isolated 12 X strains from soldiers on the West front during World War II. They did not state how long after isolation these strains had been identified. In this instance sera prepared by *Wheeler* were used.

This communication concerns three X strains of *Sh. paradysenteriae* (Flexner) which were identified two to four months after they had been isolated from faeces during an investigation of Flexner types found in Norway (*Saxholm*, 1948). Between isolation and identification the strains were kept on Dorset's egg medium which *Vogelsang* (1933) found could be relied on to preserve the antigen properties of *Salmonella* strains. Identification was carried out with type-specific sera prepared by *Weil* and also with the type-specific sera which are used by the U. S. Army laboratories. These sera were kindly supplied to us by Dr. A. J. Weil and by Dr. Ph. R. Carlquist. The U. S. Army laboratories do not produce type-specific sera for X and Y as they were not assumed to be distinct types (*Carlquist*, 1947). Using U. S. Army labo-

ratories' sera one could conclude no more than that all three strains were either X or Y. The strains were agglutinated by Weil's type-specific X serum.

Boyd (1938) has described X as an incomplete Type Z; in this connection it is worth noting that in the present investigation in addition to X several Z types were also found. However, there was no epidemiologic evidence to indicate a connection between Z and X. X was found only in scattered cases which were well isolated in time and space from the Z cases. Further, several of the Z strains were stored in the same way as X, but for a considerably longer time — over 2 years — without showing change of type.

Summary and Conclusion.

This report and others referred to show that Flexner strains of X have been identified a comparatively short time after they have been isolated. Type-specific sera from three different sources confirmed the results. Apparently, therefore, X is a valid type and not only the degenerated stock culture it is frequently taken for.

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A FAMILY ENDEMIC OF GEOTRICHOSIS PULMONUM

By Th. Thjötta and Knut Urdal.

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It is well known that certain mycoses of the lungs have attracted a great deal of interest both as distinct diseases and because of their ability of illuding as tuberculosis. This has especially been the case with Histoplasmosis, caused by *Histoplasma capsulatum* and to a smaller extent also with Geotrichosis, caused by different species of *Geotrichum*, a genus of fungi under the *Eremnascaceae imperfectae* Dodge.

While these diseases have been described quite often in American bacteriological literature, they are very seldom mentioned in the European literature. It is not clear whether these diseases do not occur in Europe or the European bacteriologists are unaware of their occurrence.

During the last autumn we happened to come across a little family endemic of Geotrichosis pulmonum, and we feel that this is a convenient occasion to give a short description both of the diseases and of the fungus in question.

The material for our investigation has been sent us from Dr. G. Sundgaard of Lillehammer who has been the physician in charge of the family. Dr. Sundgaard has for some time treated a patient staying in the home for tuberculous patients under his charge suspicious of tuberculosis, but never ascertained to suffer from this infection. Ten years ago this patient came down with a disease of her hipjoint of one side, and this was estimated to be of tuberculous origin and the joint was even operated upon. At present this patient (who is about 30 years old) still suffers from some illness of one hip joint and shows an X-ray picture very suspect of tuberculosis. The picture showed a fine infiltration scattered over the lungs especially in the middle parts and in the subclavicular space. No signs of destruction were found. While in the clinic several X-ray pictures were taken of the lungs and as the infiltrations seemed to increase somewhat Dr. Sundgaard felt the necessity of making a thorough examination of the

family of the patient. During this examination he found that a sister of the patient staying in the family home showed the same X-ray picture. Both these patients were tuberculin negative and *Mycobacterium tuberculosis* had not been found in their discharges. Under these circumstances the physician thought it most reasonable to have a bacteriological examination made, hoping to solve the problem that way. He consequently sent us the sputum from the second patient (K. M.). (fig. 1). In this sputum we found a typical strain of *Geotrichum*, species unknown, which we will describe below.

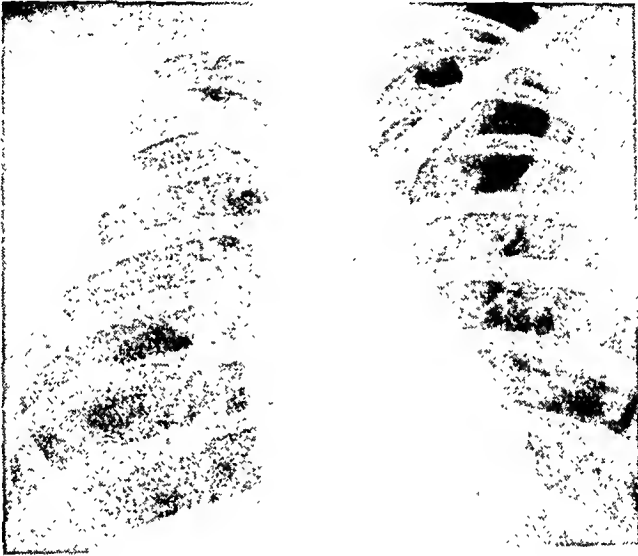


Fig. 1.

When it was clear to us that we most probably had to deal with a mycotic disease we asked for the sputa and blood proofs from the whole family of eight persons. In these sputa we found five strains of the same fungus, all strains being exactly identical morphologically, biochemically and serologically. We may therefore describe them together.

It must first be stated that only the two sisters (K. M. and G. Aa.) showed clinical symptoms of a rather mild character, some cough with expectoration of a fairly fluid sputum with distinct white, more solid flakes floating about. They had some difficulty of walking in elevated areas but had no distinct sensation of being ill and had no fever. They were also the only ones that showed the X-ray pictures described above.

In the two first expectorates we found on microscopical examination an abundance of fungous material consisting of mycelial threads and large round bodies, granulated and obviously of mycotic origin (fig. 2—3—4). We found these bodies to be chlamydospores of the fungus. In the other three specimens, however, we did not find any



Fig. 2.

Direct microscopy from K.M. $\times 150$.



Fig. 3.

Mycelial thread with chlamydo-spore. $\times 460$.



Fig. 4.

A thick clump of fungal material. $\times 400$.

mycelial material on direct microscopy, but the fungus grew out on our media as described below. In these cases we found that the sputa contained arthrospores that grew out when the expectorates were kept for some time in the laboratories. We could see the sprouting of mycelial elements from spores, when the slide preparations were examined from time to time.

This different occurrence of the fungus in the sputa is not too difficult to explain, since it is known that a fungus very often is found as spores when the expectorate is examined directly from the patient without delay, but that the mycelial elements develop when the specimen has left the patient and is kept for some time at a suitable temperature. Very likely our two first specimens had been a longer time underway from the clinic to our institute than the last specimens.



Fig. 5.
Surface colonies from Sabouraud
agar. $\times 2$.



Fig. 6.
Single colony from Sabouraud
agar. 1:1.

The cultivation of the fungus was carried out on Sabouraud agar, on Czapeks agar, malt extract agar and in fluid media of different kind. On Sabouraud agar the colonies developed within 12—24 hours and were asteroid (fig. 5) in young condition and when they grew close together. Older and single colonies showed a more or less ovoid configuration (fig. 6) with radior stripes running out from the centre giving the edge of the colony a fine crenated appearance. Often concentric rings were seen in the colony and some dense fibres running from the centre to the edge and thus giving the appearance of sectors within the colony. The colony is flat, membraneous with a dusty or velvetlike surface. The color is pure white in young colonies becoming more ivory colored as they grow older. The colony adheres to the medium, but may be emulsified quite easily. The diameter of single colonies is about 5 cm after 8 days of cultivation.

Grown on Sabouraud agar with maltose and varying pH concentrations the colonies varied somewhat in size, being largest at a higher acalinity. Thus a 4 days culture at pH 4 gave colonies of 2.1 cm. and at pH 8 2.8 cm. diameter.

On malt extract agar the colonies reached 6 cm diameter after 8 days. The colonies were otherwise as those on Sabouraud agar, but a little more yellowish of color.



Fig. 7.
Arthrospores. $\times 400$.



Fig. 8.
Sprouting arthrospores. $\times 400$.

On Czapeks agar the mycelium of the fungi grew submerged.

In fluid media a thick surface pellicle was produced, while the medium itself remained clear. On the surface of gelatine a thick pellicle was also formed and after 8 days a slight growth took place in the deeper layer of the medium.

On a close microscopical examination of the growth it was found that the fungus consisted of a mycelium of long segments or hyphae dividing into a series of rectangular arthrospores, partly arranged in chains partly torn from the chain and occurring free in the culture (fig. 7). The branching of the mycelial threads always took place immediately underneath the divisional line between two hyphae.

In order to obtain an impression of the development of the fungus from the arthrospore up to the complete fungus we inoculated Sabouraud agar plates with a thin suspension of spores and made microscopical examinations of the growth after different periods of incubation at 25° C. The spores originally inoculated were mainly cylindrical with rounded corners, while some were ovoid or spherical in structure.

After 3 hours of incubation a starting development of hyphae was observed as a slight swelling of the spore wall. This swelling was enlarged and presented itself after 4—5 hours as a cylindrical projection with a rounded end many times longer than the germ cell (fig. 8). The germination took place unipolarly and in the cylindrical spores from a corner so that the young hyphae projected from the germ cell in an obtuse angle giving the picture of a golfclub. In the ovoid and



Fig. 9.
Branching mycelium. $\times 400$.

spherical arthrospores the development of the hyphae as a rule took place perpendicularly from the cell wall. A bipolar development of hyphae might also be seen.

After 5—6 hours incubation the first division in segments was seen and after 6 hours 2—3 segments were already developed. And now a lateral branching started and practically always very close to the division of a hyphae (fig. 9). The hyphae and the branches stretched gradually in length and the outer ends were soon divided into short segments in the form of chains of cylindrical arthrospores. Seen in the colonies the segments and spores kept their places in the chain, but when emulsified the segments broke their connections and the emulsion was then mostly made up of arthrospores.

The fully developed arthrospores gradually obtained a thick doubly contoured wall, that contrasted to the thin wall of the hyphae.

The average size of the spores was $5-7 \times 8$ my, the cylindrical ones up to 13 my long and about 5 my thick, while the spherical ones had a diameter of about 6 my. On beer wort agar however the spores might gain a length of up to 30 my and give the impression of being

chlamydospores. Such big spores were seen especially in expectorate. In order to study the biochemical reactions of the two strains we inoculated them into peptone water added different carbohydrates (1 per cent) with 1 per cent of Andrade's indicator. No gas was produced, but acid was developed in glucose, levulose, and galactose, but not in mannitol, maltose, lactose, saccharose, arabinose, dulcitol, isodulcitol, xylose, inulin or salicin. The acid production was seen already after 48 hours, but grew more intense on further growth. The reading was made after an incubation during 14 days. After growth for 8 days the gelatine was liquefied from the surface downwards. Milk was not coagulated and no acid production could be found electropotentio-metrically.

No pathogenic lesion in animals was found after injection of living cultures in rabbits and guinea-pigs, intraperitoneally, intramuscularly or intravenously.

Based upon these observations of our fungus we are justified in making the diagnosis of a fungus belonging to the genus *Geotrichum*, a genus under *Fremasaccaceae imperfectae Dodge*. The type species of this genus is considered to be *Geotrichum candidum Link*, but the diagnosis of the species seems to be fairly obscure, and we have not been able to find any distinct description of any species identical to our strains, so we will leave the question of the species unsolved and only state the genus as *Geotrichum* which is quite safe as all characters of our strains are conform with the characters of that genus.

The serological examinations of the two strains were performed both in the sera from the patients and in sera produced by immunization of rabbits. The complement-fixation reaction was best suited for these examinations since the material was spontaneously agglutinable and since extracts only gave very poor precipitin-reactions. The complement-fixations were performed in the ordinary way after incubation 1 hour at 37° C. of serum dilutions with suspensions of spore-antigen from Sabouraud agar plates. Serum from patient G. Aa. gave a positive reaction up to 1 : 60 with antigens G. Aa. and K. M. while serum K. M. gave no reaction with either antigen. Serum from a rabbit immunized through 6 injections of emulsions of spore-antigen from fungus K. M. gave a positive reaction with antigens from all five strains in dilutions 1 : 640 and 1 : 320.

After these serological results it seems justified to conclude that the five strains of fungi dealt with are identical as well serologically as biochemically and morphologically. It further seems to be proved that at least one patient (G. Aa.) suffered from an infection caused by the fungus in question, since she had antibodies in her blood against this fungus. When the other patients did not show any antibody reaction towards the fungus this does not mean too much since the antibody response of patients against fungus-infections often is quite poor.

Different fungi have been described as the cause of bronchitis and bronchopneumonia. These pneumomycoses have a tendency of being very chronic. This is the case with *Geotrichosis pulmonum*. The clinical symptoms of this disease are those of a chronic bronchitis with persistent cough. The expectorate is usually white, slimy with more or less grey white flakes. Medium and coarse rales are heard over the lungs and the x-ray pictures show infiltrations and peribronchial thickenings. There may be some small cavities. As a rule the patients show only very little systemic disease and the general health is good.

The disease may simulate tuberculosis, as it has done in our cases. And this is one reason why these mycoses have gained much interest during the last years.

Geotrichosis pulmonum have not been found so frequently as *Histoplasmosis pulmonum*. Thus *Dodge* (1) find it only seldom, and *Reeves* (2) in 79 cases of pneumomycoses found it only once. Later on it has been found somewhat more frequently, so by *Smith* (3), who describes 5 cases, *de Almeida* and *da Silva* (4) four cases, *Kunstader*, *Pendergrass* and *Schubert* (5) 1 case and *Moore* (6) who describes the examinations of the fungus in a case of chronic bronchitis in a young man very minutely.

What is especially of interest in our cases is the fact that the same strain of *Geotrichum* seems to have infected several persons in the same farm and in the neighborhood of this farm. As the fungus is present in soil it seems probable that these persons are infected from the same source, maybe dust containing spores. An infection from person to person may of course have taken place, but seems less probable than dustinhalation.

As to the systematic position of *Geotrichum* this is somewhat unclear. It is quite certain that it belongs to the yeast-like fungi that forms large colonies and reproduce themselves by segmentation of the hyphae into arthrospores, as shown in our strains. According to the medical mycologist *Dodge* *Geotrichum* belongs to the *Eremasaceae* as a genus under this family. The main characteristics are as follows:

The colonies grow as fairly large, adherent membranaceous plaques on the medium. The surface is as a rule velvetlike, white or greyish white. A thick surface pellicle is formed on fluid media, gelatine is slowly liquified and some carbohydrates are digested with acid formation. The mycelium grows out from a corner of the arthrospores, a division occurs and branching sets in underneath the division lines. The edges of the hyphae are divided into rectangular arthrospores. In sputum chlamydospores may be found, but not in cultures.

Discussion.

The investigations dealt with in this paper show that it is possible to find mycoses of the lungs in our country of a clinical type that illudes as tuberculosis. In the cases treated here the fungus was *Geotrichum* of an unknown species, and the same fungus as to serological and biochemical characters were found in five out of eight members of the same family. Three of these members did not show any symptoms clinically or roentgenologically. Whether or not these three individuals really are infected or infection carriers is naturally quite unclear. The interesting question is where the fungus has been inhaled and likewise if there has been any contact infection between the members of this family. These problems have not been solved. It seems obvious that cases like ours are to be found many places if one only looks for them. It seems necessary to be on our look out, because it is rather inconvenient for patients to be listed as tuberculous patients when their infection is of another and very different kind¹).

Conclusions.

1. In five out of eight members of the same family an infection of the lungs were found to be due to *Geotrichum* of an unknown species.

2. Two of these patients had an X-ray picture that made the diagnosis of tuberculosis of the lungs probable, and one of these patients suffered from a disease of one hip joint, for which she was treated in a home for tuberculous patients.

3. All our patients had negative tuberculin reactions and *Mycobacterium tuberculosis* had never been found in their sputa.

4. It is stressed that patients like these very well may be found among patients suspect of tuberculosis.

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¹) Since this paper was written we have found 4 new strains of *Geotrichum* in sputa from patients suffering from non-tuberculous diseases of the lungs.

THE SYNERGISTIC EFFECT OF 9.10-DIMETHYL- 1.2-BENZANTHRACENE AND COLCHICINE IN SKIN CARCINOGENESIS IN MICE

By Kai Setälä.

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There are numerous suggestions in the literature that a prolonged stimulation of cell division must be present at the time of application of carcinogenic agents, or, that abundant mitotic activity occurring at the time of treatment — at least — shortens the time of onset of tumours^{1a, 5a, 5b, 6, 17a, 17b}. On the other hand, there are now known numerous chemical and physical agents which, given together with the carcinogen, cause a higher yield of tumours and/or a shortening in the latent period. These agents are called »co-carcinogenic« agents. The mechanism of co-carcinogenesis has, as yet, remained unexplained. Berenblum^{1b, 2, 3} when studying the mechanism of carcinogenesis, stated that »carcinogenesis in mouse-skin was a sequence of separate and independent processes, of which one — the preneoplastic stage of hyperplasia — was a specific response, distinct from ordinary reparative hyperplasia«.

Mottram^{17a, 17b} has concluded that three factors are involved in the production of cutaneous tumours in mice: (1) an initial »sensitizing factor«, (2) a »specific cellular reaction«, and (3) a »developing factor«. Somewhat analogous conclusions were drawn by Rous and collab.¹⁸

In an earlier paper²⁰ the occurrence of cutaneous tumours in mice treated simultaneously with the highly potent 9.10-dimethyl-1.2-benzanthracene and colchicine was preliminary reported.

The present communication deals with orientating experiments in which some solvents with different chemical and/or physical characteristics have been used as carrier for this carcinogen.

Investigation Technique.

The general investigation technique employed in the present work was essentially the same as that previously used in our experiments.^{9, 11, 23}

Because of the fact that colchicine is a very potent mitotic poison¹⁵

the highly potent 9.10-dimethyl-1.2-benzanthracene (Eastman Kodak Company, Rochester, N. Y.) was used. In the solid solvents (anhydrous lanolin, »Carbowax 1500«) the concentration was 0.25 to 0.3 per cent, and the liquid ones (acetone, dioxane) 0.03, 0.09 and 0.3 per cent respectively. The concentration of the carcinogen solubilized in aqueous »Triton NE« solution was the same as reported earlier (i. e., 0.07 per cent.)^{9, 11}.

Colchicine (Merek, Darmstadt) was dissolved in dioxane, and in aqueous »Triton NE« solution with/ or without carcinogen (0.01 to 0.1 per cent), and in »Carbowax 1500« together with the carcinogen (0.1 per cent).

The mice (unknown strain of both sexes) were treated three times weekly on a large area of the back. The solid compounds were applied without melting them beforehand.

The method of estimating the average time of response was the same used in previous experiments by the author^{10, 11, 19, 20}.

The animals were divided into subgroups, from 10 to 30 mice in each.

FINDINGS

The effect of colchicine on the intact skin.

Ludford^{14, 15, 16} has shown that the action of colchicine is a direct one upon the cells, and colchicine has the property of inhibiting the spindle formation. This has been confirmed by subsequent investigations. Cells arrested in division by colchicine present a characteristic appearance, so-called »colchicine mitosis«. It has been pointed out that in the colchicine mitosis, the cytoplasm is in »a condition of prolonged and thereby exaggerated anaphase although the chromosomes are still in metaphase«¹⁵.

The experiments with 0.1 per cent colchicine lasted about 12 weeks, during which no ulcerations (or necroses) developed. The skin of the mice showed no clear epilation either. The animals gained in weight indicating that the alkaloid was tolerated (topically applied). All the mice survived until the experimental series terminated. Microscopic examination revealed that the epidermis was considerably increased in thickness (Fig. 4). The basal cells were prominent, with large (sometimes clumped) nuclei, but scanty cytoplasm, resembling the »podophyllin-« and/ or »colchicine cells« reported by e. g., King and collab.^{12, 13}. Also the kerato-hyaline granules in the superficially situated cells of the epidermis were greatly accentuated. The epidermis was, in general, covered by a thin, laminated, horny layer. There was no evident destruction of the sebaceous elements. The subcutaneous connective tissue layer was somewhat thickened, but there were no distinct signs indicating inflammatory changes.

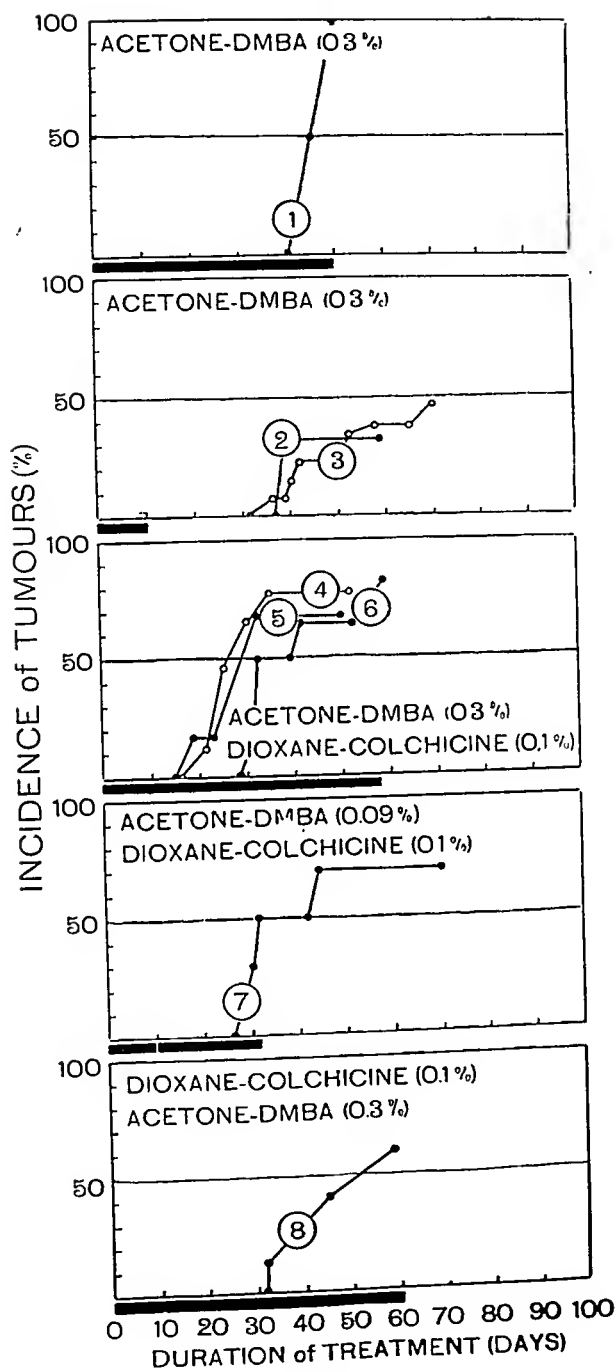


Fig. 1.

Incidence of tumours in relationship to the additional treatment. The carcinogen dissolved in acetone, and colchicine in dioxane respectively.

Abcissae: duration of treatment (days),
Ordinates: incidence of tumours (per cent).

The experiments with 0.01 per cent colchicine lasted also about 12 weeks. In some cases there was no reaction at all. However, in most cases the occurrences described above were encountered, although weakly.

The effect of colchicine applied to the skin together with the carcinogen.

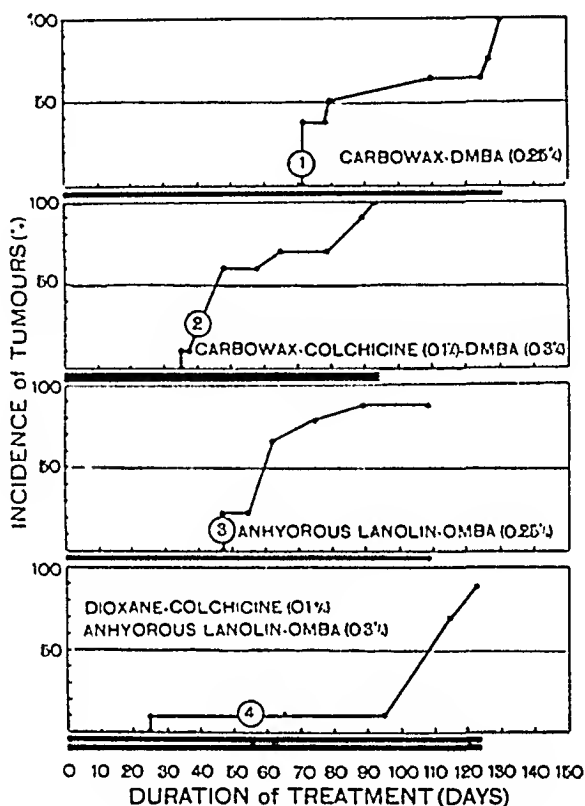


Fig. 2.

Incidence of tumours in relationship to the additional treatment. »Carbowax 1500«, and anhyorous lanolin as solvents for the carcinogen. Colchicine dissolved in dioxane, and in »Carbowax 1500«.

Acetone as solvent for the carcinogen (Fig. 1). — In the following paragraphs only some of the experiments are reported.

(1) Acetone controls (0.3, and 0.03 per cent carcinogen), without additional colchicine treatment. The average time of response was 6½ and 7 weeks respectively.

(2) 0.3 per cent carcinogen dissolved in acetone painted for 10 days (five applications), without additional colchicine treatment. During the experimental period the incidence of tumours did not reach the figure of 50 per cent (Fig. 1½-3).

(3) 0.3 per cent carcinogen dissolved in acetone painted for 10 days (five applications), followed by treatment with 0.1 per cent colchicine: by the 2nd and 3rd weeks there was a distinct epilation in all animals. Regrowth of the hair began later, and the skin of all mice was fully haired when the experiment terminated. The first warts appeared in the beginning of the 3rd week. The average time of response varied from $3\frac{1}{2}$ to $4\frac{1}{2}$ weeks (in different series). The incidence

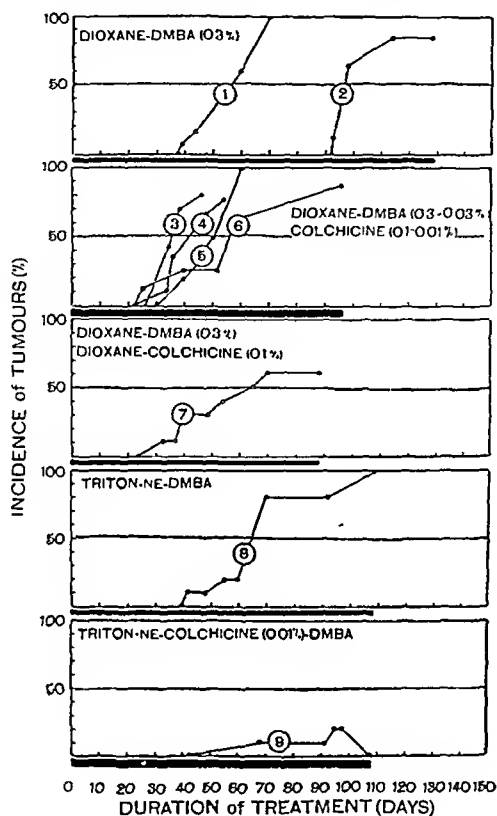


Fig. 3.

Incidence of tumours in relationship to the additional treatment. Carcinogen and colchicine dissolved simultaneously in dioxane, and in aqueous »Triton NE« solution respectively.

of tumours reached the figure of c:a 85 per cent (Fig. $\frac{1}{4,6}$, and Fig. $\frac{1}{5}$).

(4) 0.09 per cent carcinogen dissolved in acetone painted for 10 days (five applications), followed by treatment with 0.1 per cent colchicine: the skin was completely epilated by the 2nd week of the experiment. However, there was a distinct regrowth of the hair in all animals. The average time of response was about $4\frac{1}{2}$ weeks, and the incidence of tumours reached the figure of 70 per cent (Fig. $\frac{1}{7}$).

(5) 0.1 per cent colchicine dissolved in dioxane painted for 10

days (five applications) followed by treatment with 0.3 per cent carcinogen dissolved in acetone: the average time of response was about $7\frac{1}{2}$ weeks (estimated from the beginning of the treatment with the carcinogen). The incidence of tumours was about 60 per cent (Fig. $\frac{1}{8}$).

(6) 0.3 per cent carcinogen dissolved in acetone and painted for 7 days (three times), followed by treatment with 0.1 per cent col-



Fig. 4.

Mouse skin treated (11 weeks; three times weekly) with 0.1 per cent colchicine dissolved in dioxane. Numerous cells, resembling the «podophyllin cells» by King, in the basal layers. Thickening of the epidermis.

chicine: the first solitary growing warts appeared by the 4th week of the experiment. There was also a weak epilation in some cases. All tumours disappeared, however, after a short time.

(7) 0.3 per cent carcinogen dissolved in acetone and painted once only, followed by treatment with 0.1 per cent colchicine: there developed only a single wart which disappeared rapidly.

(8) 0.03 per cent carcinogen dissolved in acetone, and painted one, three or five times respectively, followed by treatment with 0.1 to 0.01 per cent colchicine dissolved in dioxane: there developed single warts in each group, but the tumours disappeared, however, rapidly.

(9) 0.3 per cent carcinogen dissolved in acetone, and painted once only, both preceded and followed by treatment with 0.1 per cent colchicine: no tumours developed.

(10) 0.03 per cent carcinogen dissolved in acetone and 0.1 per cent colchicine dissolved in dioxane painted in turn (three times weekly) over a long period of time: no tumours developed.

«Carbowax 1500» as solvent for the carcinogen (Fig. 2). — The following experiments are reported:

Sept. 4, 1948.

5 applications of DMBA (0.3%) in acetone.
"Post-treatment" (10 applications) with
dioxane-colchicine (0.1%). 3 x wly.



Fig. 5.

Multiple warts, without epilation of the skin.

Sept. 4, 1948.

44 applications (3 x wly) of DMBA (0.3%)
in "Carbowax-1500"-colchicine (0.1%).



Fig. 6.

Multiple warts appearing during treatment with »Carbowax 1500« containing simultaneously both carcinogen and colchicine.

(1) 0.25 per cent carcinogen dissolved in »Carbowax« and applied continuously without additional treatment with colchicine: the average time of response was about 11½ weeks. The incidence of tumours was 100 per cent by the 18th week of the experiment (Fig. 2/1).

(2) 0.3 per cent carcinogen and 0.1 per cent colchicine dissolved in »Carbowax«, and applied simultaneously: by the 2nd and 3rd week of experiment there were signs indicating the beginning of epilation. The average time of response was about $6\frac{1}{2}$ weeks. The incidence of tumours reached the figure of 100 per cent by the 13th week (Fig. $\frac{2}{2}$, and Figs. 6 and 7).

Anhydrous lanolin as solvent for the carcinogen (Fig. 2). — The following experiments are reported:



Fig. 7.

Biopsy specimen from tumours seen in Fig. 6.

(1) 0.25 per cent carcinogen dissolved in anhydrous lanolin, and applied continuously without colchicine treatment: the average time of response was about $8\frac{1}{2}$ weeks. The incidence of tumours reached the figure of 90 per cent by the 13th week (Fig. $\frac{2}{3}$).

(2) 0.3 per cent carcinogen dissolved in anhydrous lanolin and painted for 10 days (five times), followed by treatment with 0.1 per cent colchicine: neither tumours nor epilation developed.

(3) 0.3 per cent carcinogen dissolved in anhydrous lanolin applied together with 0.1 per cent colchicine dissolved in dioxane over a long period of time: by the 2nd week there were signs indicating initial epilation. The first solitary growing wart appeared by the 3rd to 4th week of the experiment. The number of tumours-bearing mice increased, however, first by the 14th week of the experiment. The average time of response was about $15\frac{1}{2}$ weeks, *i. e.*, nearly twice as long as that reported in the paragraph 1 (Fig. $\frac{2}{4}$).

(4) 0.1 per cent colchicine dissolved in dioxane and painted for 10 days (five applications), followed by treatment with 0.3 per cent

carcinogen dissolved in anhydrous lanolin: no distinct epilation at all developed. The average time of response was about 15 weeks.

»Triton NE« solution as solvent for the carcinogen (Fig. 3). — Two experiments were made:

(1) The carcinogen was applied continuously without additional colchicine treatment: the average time of response was about 9 weeks. All the animals had tumours by the 16th week of the experiment (Fig. 3/s).

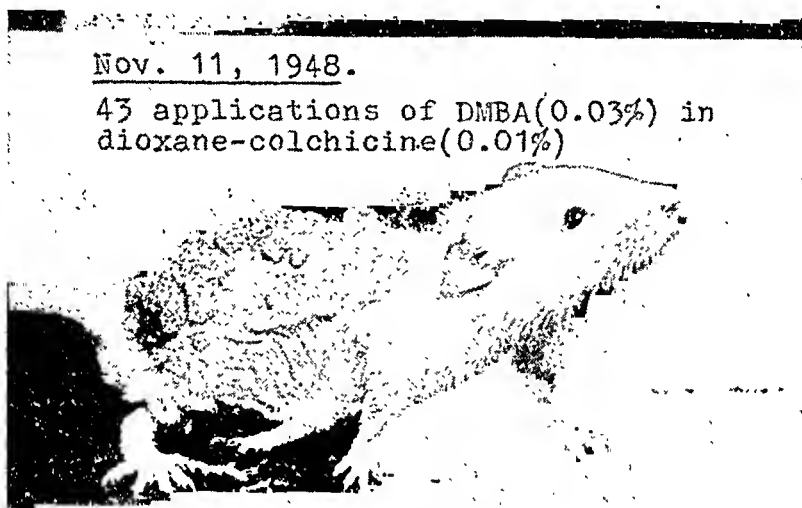


Fig. 8.

Mouse treated 43 times simultaneously with the carcinogen and colchicine.

(2) The carcinogen was applied continuously with additional colchicine. The first experiment: »Triton NE« solution contained simultaneously 0.07 per cent carcinogen and 0.1 per cent colchicine. The epilation began by the 3rd week of the experiment. About 14 weeks after the beginning of the experiment all mice were epilated. The addition of 0.1 per cent colchicine inhibited the development of tumours.

The second experiment: the aqueous »Triton NE« solution contained simultaneously 0.07 per cent carcinogen and 0.01 per cent colchicine. The epilation was not so distinct as in the foregoing paragraph. The incidence of tumours reached the figure of 20 per cent by the 14th week of the experiment. However, after this time the tumours disappeared (Fig. 3/o).

Dioxane as solvent for the carcinogen (Fig. 3). — The following experiments are reported:

(1) 0.3 per cent carcinogen dissolved in dioxane and painted continuously without additional colchicine treatment: the incidence of

tumours as well as the epilation of the skin varied considerably in different experiments (depending upon the degree of purity of the compound and/or upon the percentage of water in dioxane). The average time of response varied from about 8 weeks to about $13\frac{1}{2}$ weeks respectively (Fig. $3/1-2$).

(2) 0.03 to 0.3 per cent carcinogen dissolved in dioxane and painted together with 0.01 to 0.1 per cent colchicine (several experimental series): the higher the concentration of the carcinogen the earlier symptoms indicating epilation of the skin appeared. The circumstances were the same as regards the initial period and the average time of response. Thus, the shortest average time of response was about $4\frac{1}{2}$ weeks, and the longest one 8 weeks (Fig. $3/3-6$). The tumours appeared, in general, as multiple growing warts (Fig. 8).

(3) 0.1 per cent colchicine dissolved in dioxane and painted for 10 days (five applications), followed by treatment with 0.3 per cent carcinogen dissolved in dioxane: the average time of response was about 8 weeks (estimated from the beginning of the treatment with carcinogen (Fig. $3/7$).

Discussion.

Besides the significance of cell division in experimental carcinogenesis, there are also several other factors influencing the onset of cutaneous (as well as other) tumours. Thus, the *nature of solvents* plays an important role in the carcinogenic response of the tissues. On the other hand, numerous »additional« agents (chemical and physical) are now known to have an augmentative or inhibitory effect on tumour production. Berenblum² has summarized the experimental work on the agencies which retard, accelerate or leave unaffected the carcinogenic action of tar and polycyclic hydrocarbons. The mechanism of this action has, however, remained unexplained. Berenblum^{1b} gives the following possibilities: first, that the co-carcinogenic action (of croton resin, a potent co-carcinogen) may be that it is merely a variant of epi-carcinogenic action, on the supposition that the dilute carcinogen produced the preneoplastic state, while the epi-carcinogenic effect is carried out jointly by the dilute carcinogen and the co-carcinogenic agent (croton resin). Second, that co-carcinogenic agent (croton resin) merely facilitates the entry of the carcinogen into the cell so that a small number of molecules of the hydrocarbons, applied to the surface, would still have a reasonable chance of acting on the cell.

In the experimental use of colchicine the drug has rarely been applied (in the literature) directly to the skin. Sometimes, however, it has been applied topically as oil suspensions^{12, 13a, 13b}. In the present work colchicine was applied topically dissolved in dioxane, and in »Carbowax 1500«, and solubilized in aqueous »Triton NE« solution

(the last mentioned is an association colloid^{9, 11}). It appeared that there developed neither ulcerations nor a permanent epilation of the skin when colchicine was used without carcinogen. When, on the contrary, colchicine was used as additional treatment during or after treatment with the carcinogen, the effect of colchicine seemed to be dependent upon several factors modifying the results.

Among these factors the chemical and/or physical character of the solvent (*i. e.*, »carrier«) seemed to be a very important one. But, however, the nature of this »solvent effect« is still almost unexplained. Because of this, the importance of the »solvent effect« must be clear before the quantitative investigations with added material (*e. g.*, colchicine) are continued.

All the observations presented here, are now under reexamination and quantitative study. Many facts have already been confirmed, but it is, as yet, too early to report detailed results.

Because of the wide scope of the question we do not want to discuss the problem in this connection. There are, as shown in the present report, several still *unknown* factors modifying the results, that is also why the application of statistical methods in this stage of the experiments would be incorrect.

Summary.

Data have been presented which indicate that the carcinogenic response of the mouse skin varies considerably, when colchicine and 9.10-dimethyl-1.2-benzanthracene act synergistic, depending upon several factors.

The significance of the so-called »solvent-effect« of the carrier for the carcinogen is emphasized.

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AN ANTIBIOTIC EFFECT INHIBITING THE COLI-AEROGENES GROUP IN THE INTESTINES OF GUINEA-PIGS

(Preliminary report)

By *Gunnar Fischer.*

(Received for publication February 14th, 1949.)

The faeces of a large number of guinea-pigs have been investigated by cultivation on Endo- and bromthymol blue agar. There was no growth of coli-aerogenes in the majority of cases, but from a few guinea-pigs a colony or two could be cultivated. Some of the guinea-pigs were dissected and a great number of samples were taken from the cecum down to the anus. As a rule no coli-aerogenes growth was obtained, but in a few cases some colonies of coli-aerogenes could be cultivated.

On the other hand, in rabbits, an abundant growth of coli-aerogenes was obtained along the whole tract from the cecum to the anus. As the breeding conditions of the two kinds of animals were identical, it would seem probable that one or more factors exist in the intestines of the guinea-pig, which have an unfavourable effect on the development of coli-aerogenes. The following tests were therefore performed:

The hydrogen-ion concentration in the intestines of the guinea-pigs from the cecum to the anus was determined. It was found to be about 7 and, accordingly, could have no unfavourable effect on the development of coli-aerogenes.

For the purpose of detecting the antibiotic factor, the intestinal flora was cultivated on agar-plates as follows:

A culture of *b. coli* was grown in broth for 20 hours and diluted with broth in the proportion 1:10; 0.15 ml of this dilution was added to 20 ml melted meat-agar, and carefully mixed. A portion sufficient for one plate was then poured into a Petri dish. When the plate had solidified, pure cultures of the intestinal bacteria, whose antibiotic effect was about to be studied, were streaked on small spots of the surface. The plate was subsequently incubated at 37° for 24 hours. Should an antibiotic effect exist, inhibition of the growth of the coli bacteria would be perceptible either under or round the streaked material; in the latter case a clear zone surrounding the antibiotic material would be seen.

In performing the tests, a number of well-known laboratory strains of the coli-aerogenes group were employed. Agar media containing serum, ascites and plasmolysed blood were used. The tests were carried out under both aerobic and anaerobic conditions ad modum Fortner.

A large number of micro-organisms, cultivated from different parts of the intestines were tested. Some of these were sarcinae, cocci, aerobic spore forming bacteria as well as various kinds of moulds. A gram-positive coccus was the only bacteria which manifested an antibiotic effect; a very pronounced antibiosis was revealed against the majority of the investigated coli-strains. A well-defined clear zone of 3—5 mm surrounded this bacteria in both aerobic and anaerobic cultures. In tests with staphylococci and salmonella typhi murium, it was found that this micrococcus had an even more strongly marked antibiotic effect against these test bacteria.

The micrococcus was found to have the following properties:

It is gram-positive and has a diameter of about 0.9—1.3 μ .

It grows on ordinary agar, whether aerobic or anaerobic, with small crystal-clear colonies 0.2—0.3 mm in diameter. It also grows well in broth and always produces a uniform turbidity. In fermentation-tests the results were as follows: It ferments glucose, rhamnose, lactose, maltose and saccharose with the production of acid but not gas.

A very abundant growth of micrococci of this type was found in the rectum of every guinea-pig. Only some of the cultivated strains, however, manifested an antibiotic effect. This effect is still being studied and will be the subject-matter of a future publication.

INTRACELLULAR PROTEIN CRYSTALLIZATION

By Lennart Zettergren.

(Received for publication March 7th, 1949.)

Alongside of the physiologic occurrence of crystalline protein in the interstitial cells of the testicle, well known since long ago, protein crystals have been observed, though extremely seldom, also in other human tissues. All the cases of intracellular protein crystallization reported in the literature seem to have disclosed a connection between this phenomenon and either typical »inflammatory« or atypical plasma cells.

Firstly, as regards the crystallization in *atypical plasma cells* (plasmocytoma cells), *Glaus*, in 1917, described a case of plasmocytoma where peculiar, partly crystal-like, strongly refractive formations were observable in the cytoplasm of the tumor cells. To judge from the illustrations in his work, some of the *tumor cells* were enlarged and filled with collaterally stored, elongated, narrow structures. In 1919, *Steinmann* reported a case of a leukemic plasmocytoma where the tumor cells obtained from the blood and by sternal puncture contained, apart from thin fusiform rods with tapering ends, also crystalloid rhomboedric bodies. Finally, *Apitz* (1941) presented two cases of plasmocytoma with intracellular protein crystals. In one of these cases, approximately one plasma cell out of 30—40 was found to be enlarged and filled with up to a hundred small, oval or rounded bodies. These bodies were markedly eosinophile and, at Weigert's staining method, assumed a strongly dark blue colour, while the azan or Masson's stain gave them an intense red colour. Among the amyloid reactions, only the one with Congo red became positive. In polarized light the inclusions were faintly, but definitely, refractive. It is noteworthy that similar crystalline protein accumulations were observed in the epithelial cells of the renal tubules in the same case. In *Apitz'* other case, an abundance of collaterally stored, acicular crystal formations with the same tinctorial properties as in the former case were seen in a few very large tumor cells.

However, as mentioned by way of introduction, cases have been

described where crystals of a protein nature have been found in ordinary »inflammatory« *plasma cells*. Thus, in 1889 *Mibelli* noticed, in his studies of rhinoscleromas, that the hyalin substance in some »hyalin cells« fell into rod-shaped fragments, »einem Zigarrenbunde ähnlich«. He interpreted these inclusions as scleroma bacilli contained in the cells, while *Apitz*, among others, was convinced that they referred to protein crystals. In 1896, *Podwyssotzky* stated that he had, in a case of maxillary cancrroid that involved a pronounced granuloma formation, among the cells in the granulation tissue observed some containing hyalin structures in the cytoplasm. Some of these bodies had a globular shape, others were characterized as rhombic crystals. In 1913, *Helena Freifeld* described a similar case. This was a matter of an adenocarcinoma in the maxilla. Apart from Russell's bodies in large quantities, she found in the granulation tissue pervading the stroma of the tumor »die Zellen mit einer Anzahl von länglichen, rhombischen Kristallen ausgefüllt, die meistens einander parallel lagen und alle Eigenschaften von Hyalin behalten hatten«. *Van den Berghe*, in 1947, reported that he had observed, in the bone marrow of the rabbit, four plasma cells with crystalloid cytoplasm inclusions, and likewise in the bone marrow of a 4-year-old child one single plasma cell with similar inclusions. However, van den Berghe did not offer any data regarding the optical activity or tinctorial properties of the assumed crystals.

Since the majority of the observations presented in the literature regarding the intracellular protein crystallization date far back and, in some respects, differ from a case ascertained and closely studied by the author, it seems justifiable to give a brief account here of this puzzling phenomenon.

E. M., aged 54. For several years, tinnitus in the left ear. For this reason, applied in May 1948 at the Ear Clinic of the Sollefteå Hospital (Hosp. rec. num. 1894/48). *Physical exam.*: From the left tympanic membrane a granulating polypus projects, the size of a pea and resembling a tumor. Evulsion. *Microscopic exam.*: The surface of the polypous tumor is provided with an occasionally ulcerated, thin, squamous epithelial coat. Under the epithelium, a fairly broad zone of granulation tissue extends with masses of typical plasma cells. Then follows a region with a fibrous connective tissue, relatively deficient in cells, that delimits the central parts of the tumor and consists of a faintly coloured tissue in alveolar arrangement (Fig. 1). The tumor cells are fairly rich in cytoplasm, while the nuclei poor in chromatin are, as a rule, rounded or oval and vary but slightly in size. No mitoses are observed. The slender stroma is notably rich in sinusoid capillaries. The tumor conforms, as regards its histologic structure, to the type of middle ear tumors referred to under the name of »tumor of carotid body type« and described by *LeCompte* and coworkers, among others, being assumed to issue from the so-called glomus jugularis (*Guild*).*)

*) A more detailed account of the tumor will be given in another connection by Dr Nils O. Berg, Lund.

In the beginning of June 1948, a new polypus had formed. It had approximately the same localization and macroscopic appearance as the previously removed one. The patient disclosed an effusion from a perforation in the anterior part of the tympanic membrane. Evulsion of the polypus. *Microscopic exam.*: The tissue has, on the whole, the same appearance as at the previous examination, though being more loose and rich in vessels than before.

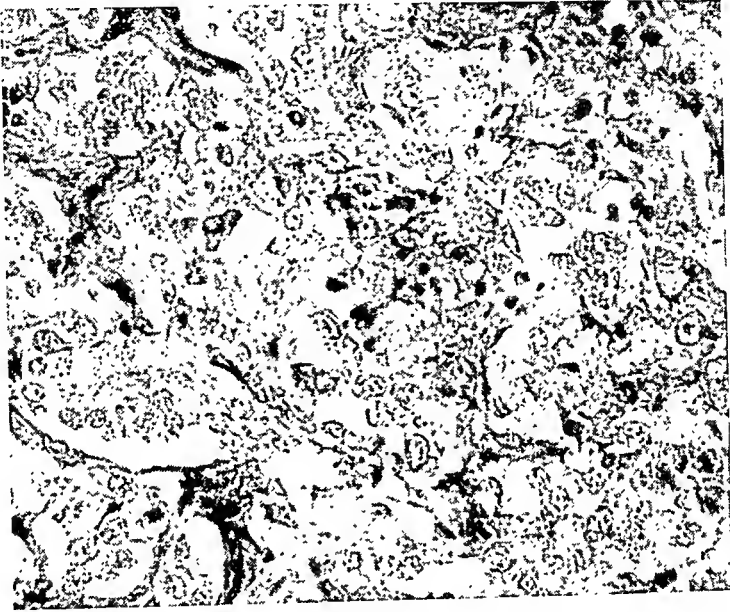


Fig. 1.

A section of the tumor showing its alveolar structure here and there.
v. Gieson. $\times 250$.

In the middle of June a *radical operation* was performed (Kyhlestedt): Large cell system filled with discoloured fluid. Along the prominence of the facial canal near the antrum, small polypi were seen. The whole of the middle ear was filled with granulations, some of which were transmitted for patho-anatomic diagnosis. *Microscopic exam.*: On the whole, the same histologic picture as at the previous examination.

However, in the material first subjected to examination, the most remarkable finding is the occurrence of peculiar distinctly quadrate, strongly refractive inclusions in some of the cells of the granuloma. These particular cells are more or less enlarged, evidently, approximately in direct proportion to the size of the inclusions. In the cases where the inclusion takes up the greater part of the cell, the nucleus is seen to be flattened and pressed against the periphery of the cell.

The cells supplied with inclusions are principally encountered within the parts of the granuloma where the plasma cells are most numerous, i. e. in the subepithelium. At a closer study of the cells containing minor inclusions and, consequently, essentially retaining their original structure, it will be observed that these cells have an appearance characteristic of the plasma cell. Thus, the nucleus of the cells is, as a rule, eccentrically situated with a conspicuous border position of the chromatin and a perinuclear clarification in the basophile cytoplasm.

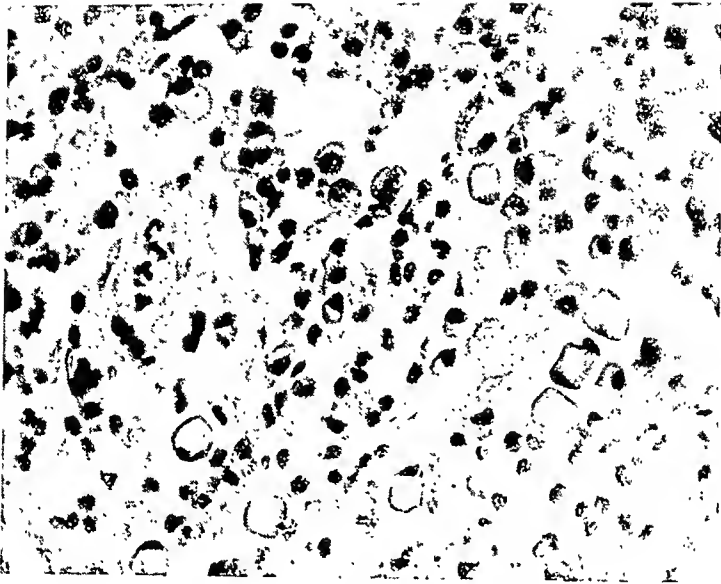


Fig. 2.

A section of the granulation tissue showing a number of plasma cells provided with inclusions. v. Gieson. $\times 250$.

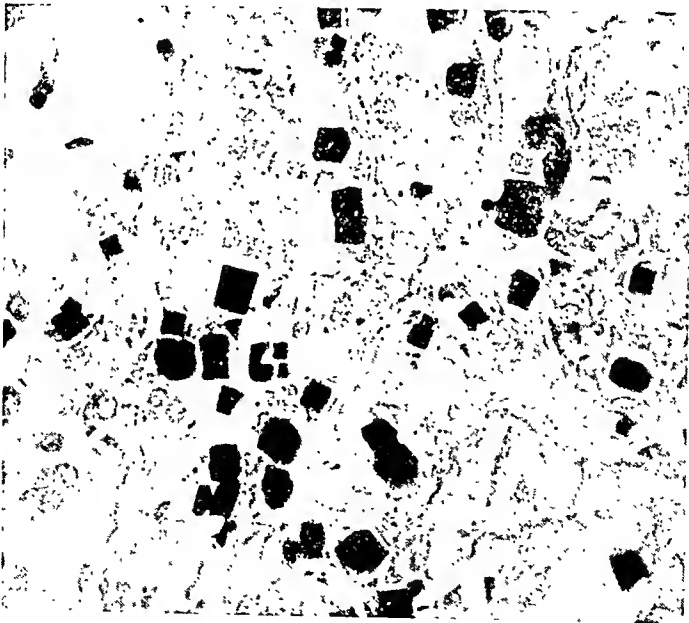


Fig. 3.

Azan-stained preparation with a number of protein crystals of varying size. The deviation from the quadrate form that may be seen in places is partly due to a varying direction of the cuts through the cubic crystals, and partly to the position of adjacent crystals in different planes of the preparation. $\times 300$.

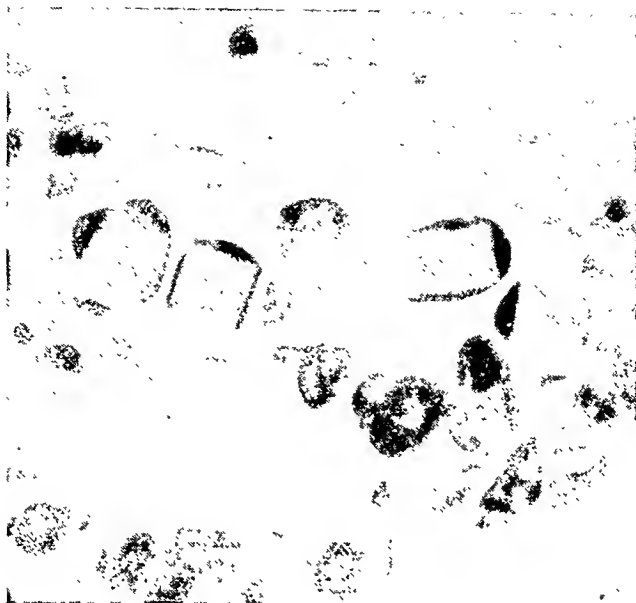


Fig. 4.

Some crystals one of which (the second from the left) has been cut approximately parallel with one of the surfaces of the cube. v. Gieson. $\times 700$.

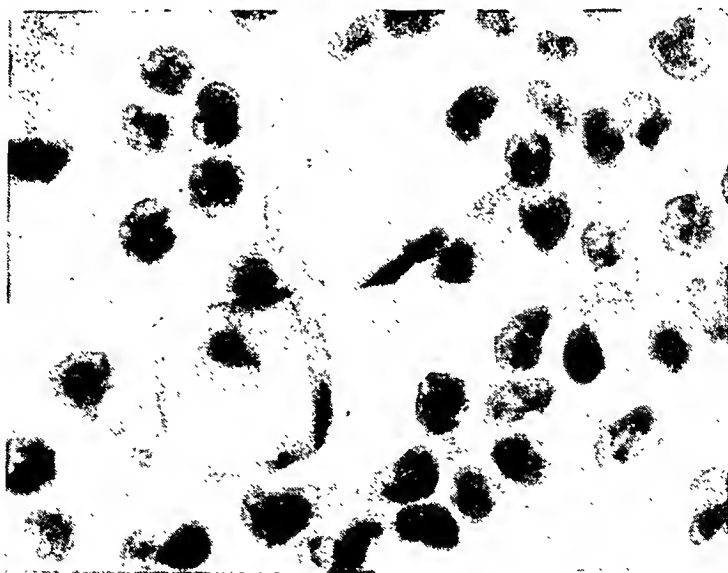


Fig. 5.

Two crystals, the upper one viewed against one of the edges of the cube. v. Gieson. $\times 1000$.

The inclusions which are strongly refractive are on closer examination found to be cubic. This is proved by the fact that inclusions sectioned in a plane parallel with one of the surfaces of the inclusion will always be quadrate. Their size varies within fairly wide limits. The smallest specimens will measure only $1\ \mu$ and the biggest will have a border length of about $20\ \mu$. Accurate measurements of a comparatively large number of inclusions show that about 50 per cent have a border length varying between 12 and $16\ \mu$.

Generally, there is only one large inclusion in each cell, but a few smaller ones in isolated cells. However, not even the biggest inclusions fill up the cytoplasm entirely, but only come in contact with the cell membrane at their corners, while this is otherwise separated from the surfaces of the quadrate by a thin layer of cytoplasm (Fig. 5). This contributes to the often rounded quadrate appearance of the cells supplied with inclusions, as observed at moderate magnification in preparations stained with eosin or van Gieson's solution (Fig. 2).

From a polarimetric point of view, the inclusions do not disclose any anisotropy. However, since their refraction index exceeds that of the surrounding parts (= Canada balsam), they are easily detectable also in unstained preparations.

As regards the staining, the inclusions have the following properties. At staining with hematoxylin-eosin they assume a pale red tint, at van Gieson's staining method a yellow one. In plasma cell preparations stained according to the Unna-Pappenheim method, they appear bluish-grey-violet, with Congo red they become pale red, and with azan intensely red. Weigert's fibrin staining, finally, gave inclusions of a blue-black colour. Amyloid staining with methyl-violet was negative. Unfortunately, no material was available for fat staining or chemical analysis.

Discussion.

Since intracellular protein crystallization is no doubt a very rare phenomenon, it seems justified to make a closer study of the problem of whether the formations described here are actually to be interpreted as intracellular protein crystals. The following questions present themselves:

- 1) Are the formations intracellular?
- 2) Are they of a protein nature?
- 3) What factors speak in favour of their crystal nature?

Ad. 1. The question whether the presumed crystals are intracellular does not have to be further discussed. This is clearly evident from Figs. 4 and 5. Nor does any doubt exist as to the particular type of cells in which they have formed, for, as already pointed out, the cells provided with the smaller inclusions disclose an appearance typical of the plasma cell.

Ad. 2. That the inclusions are of a protein nature is demonstrated by the outcome of the staining reactions. In a case like this it would, of course, be of great interest to obtain a more detailed conception of the chemical nature of the protein. However, this is apparently not

possible by means of tinctorial methods. As shown by *Apitz*, concerning the question of the protein (paraproteins) in cases of plasmocytoma, it is not the chemical structure but the colloidal form of the protein that is decisive for the outcome of the staining reactions.

Ad. 3. From a polarimetric point of view, no direct support is found for the crystal nature of the inclusions in the form of anisotropy. However, it has been pointed out earlier that the inclusions in this particular case are cubic. Accordingly, they belong to the regular crystal system (a crystal system with three equal axes perpendicular to each other). As to the crystals appertaining to this system, they do not manifest anisotropy. Thus, merely the cubic form of the inclusions, in combination with their strong refractive power, justifies their classification as crystals.

The question now arises why cytoplasmic protein in this case changed to a crystalline form. Apparently, no definite answer can as yet be given. However, it should be borne in mind that the occurrence of the protein substance in a crystalline form is a criterium of its chemical purity.

Cases with crystalline protein inclusions in plasma cells bring to the fore a number of problems of vital interest, particularly considering the relation of the plasma cell to certain pathologic protein substances of antibody nature. As early as in 1913, *Huebschmann* stressed the possibility that the plasma cell might have a capacity of forming antibodies. However, *Bing & Plum* were the first to ascertain the regular occurrence of hyperglobulinemia simultaneously with a more extensive proliferation of the plasma cell. *Bjorneboe & Gormsen* demonstrated that, after immunization of rabbits with pneumococci, hyperglobulinemia and plasma cell formation occurred at the same time in diverse organs. According to the last-mentioned authors, the connection between pathologic globuline and the plasma cell increase may be explained in three different ways:

1) The globulin increase and the plasma cell increase have a common cause, but are otherwise only parallel phenomena.

2) The plasma cell formation is secondary to the hyperglobulinemia in so far as the latter stimulates the plasma cell formation, or the new-formed globulins are stored in the plasma or in their early stages, analogously to the condition manifested at the so-called thesaurismoses.

3) The plasma cells form the globulins or certain fractions of them.

The problem that in the present author's case is most noteworthy is whether the occurrence of protein crystals is a manifestation of a protein-forming capacity of the plasma cell, or of a resorptive property in these cells (thesaurismosis). The investigations performed by *Astrid Fagraeus* are of great value for an estimation of this matter.

Fagraens demonstrated that reticulo-endothelial cell elements will, under certain experimental conditions, produce antibodies and thereby, via so-called transitional cells and immature plasma cells, transform into a cell type with the morphologic characteristics of the plasma cell. In the light of these observations, it seems exceedingly improbable that intra-cellular crystalline protein is composed of protein resorbed in the cell. On the other hand, it is conceivable that the plasma cells (transitional cells, immature plasma cells) in the preliminary stages, for reasons unknown to us, were incapable of discharging the antibody protein which was, therefore, stored intracellularly and — possibly owing to a chemically uniform and pure composition — there assumed a crystalline form. In that case, the crystals may be supposed to consist of some kind of globulin.

Finally, the question may be asked whether the crystals described above are related to the so-called Russell's bodies which also appear in plasma cells and disclose the same tinctorial properties as the crystals. However, no definite transitional forms between the very sparse Russell's bodies and the crystals have been ascertainable.

Summary.

A case of intracellular protein crystallization is reported in a tumor deriving from the tympanic membrane, of a so-called xanthoid body type (Le Compte and coworkers). The crystals formed in typical inflammatory plasma cells, generally one in each cell, are cubic and strongly refractive but do not disclose any anisotropy. Their size varies within fairly wide limits, but about half have a border length of between 14 and 16 μ . They show the following tinctorial properties: At staining with hematoxylin-eosin, they assume a pale red colour, with van Gieson's stain a yellow tint. In plasma cell preparations stained according to the Unna-Pappenheim method, they become bluish-grey-violet, and with azan intensely red. The amyloid reaction with methyl-violet was negative. Finally, Weigert's fibrin stain gave the inclusions a blue-black colour.

The author discusses the question of whether the crystallization is a manifestation of a resorptive, so-called thesaurismotic property in the plasma cells, or of a protein-producing capacity in these cells. The possibility that a chemically uniform structure in the intracellularly formed protein is the cause of this crystallization is pointed out.

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THE GROWTH CAPACITY OF CORYNEBACTERIUM DIPHThERIAE GRAVIS, MITIS AND INTERMEDIUS AT HIGHER TEMPERATURES

By Per Oeding.

(Received for publication June 22nd, 1919.)

It was demonstrated by Weiland and Leinbrock (1938) that *C. diphtheriae gravis* was slightly more resistant against heating and disinfectants than *mitis*. All the 5 *gravis* strains examined were viable after 5 minutes in water bath at 56°, whereas the 5 *mitis* strains were dead and the *intermedius* strains took an intermediate position. No examinations of the capacity of the 3 types to grow at higher temperatures than the optimum have been published.

Experimental.

The optimum temperature for the cultivation of the diphtheria bacillus is 36—37°. It also grows at 20° on ordinary media but at that temperature the growth is more scanty and slow than under normal conditions, and the colonies lose their typical appearance. At 45° the diphtheria bacillus does not grow, but it was found to be a distinct difference between the growth capacity of the 3 types at higher temperatures than the optimum. These findings suggested a closer examination on this subject, because the preliminary examinations indicated that this might be a reliable method by which the types could be distinguished.

The growth capacity of 6 *gravis*, 6 *mitis* and 2 *intermedius* strains were examined in an incubator at 45°, 44°, 43°, 42°, 41°, 40°, 39° and 37°. From 24 hours old Loeffler slants 1 loopfull of the condense water was simultaneously transferred to a tube with phosphate broth and a tellurite medium (Hoyle's medium). 10 mm wide tubes

Table 1.
The growth capacity of the 3

Strains			Phosphate										
			44°			43°			42°			41°	
			1	2	3	1	2	3	1	2	3	1	2
Mitis	F 86		0	0	0	0	0	0	0	0	0	0	0
-	F 371		0	0	0	0	0	0	0	0	0	0	(+)
-	F 397		0	0	0	0	0	0	0	0	0	0	0
-	F 432		0	0	0	0	0	0	0	0	0	0	0
-	F 4166		0	0	0	0	0	0	0	0	0	0	0
-	F 4585		0	0	0	0	0	0	0	0	0	0	0
Gravis	F 132		0	0	(+)	0	(+)	(+)	(+)	(+)	(+)	0	0
-	F 133		0	0	(+)	±	(+)	(+)	±	±	(+)	±	±
-	F 134		0	0	(+)	0	(+)	(+)	+	+(+)	+(+)	+	+
-	F 357		0	±	(+)	0	(+)	(+)	(+)	(+)	(+)	(+)	+
-	F 364		0	0	±	(+)	+	+	+	+	+	(+)	+(+)
-	F 435		0	0	±	+	++	++	+	+	+	0	+++
Interm.	F 3764		0	0	±	±	(+)	(+)	(+)	(+)	(+)	(+)	+
-	S 499		0	0	0	0	0	0	±	±	±	+	+

Controls 37°: +++

The results are read after 1, 2 and 3 days. The growth is graded from 0 = no

containing 3 ml phosphate broth were used. On each Hoyle plate 6 strains were inoculated in sectors. At each temperature examined, controls were incubated at 37°. The results were read after 1, 2 and 3 days. The temperature of the incubator was thoroughly controlled at each new adjustment.

It will be seen from table 1 that gravis is able to grow at higher temperatures than mitis. At 44° 4 of the gravis strains showed a faint growth, and at 43° all 6 gravis strains were able to grow. Only at 41° did 2 of the mitis strains exhibit a faint growth, and all mitis strains grew at 39°. Intermedius took an intermediate position between gravis and mitis. Similar results were obtained on Hoyle's medium, where the growth started at a slightly lower temperature than in broth. At 20° the growth was atypical, but no difference between the growth energy of the 3 types could be demonstrated.

The pronounced thermo-lability of the mitis type was visible as low as at 37°, as well in phosphate broth as on Hoyle's medium. It is therefore possible that the mitis strains which by some authors have been found to grow badly on tellurite media at 37°, may have been particularly thermo-labile strains.

40° was the temperature best suited for the division between gravis

types at higher temperatures.

broth cultures

40°			39°			37°			
3	1	2	3	1	2	3	1	2	3
0	(+)	(+)	+	++	++	++	+++	+++	+++
(+)	0	$\frac{1}{2}$	+	+++	+++	+++	+++	+++	+++
0	0	0	(+)	+++	+++	+++	+++	+++	+++
(+)	0	$\frac{1}{2}$	(+)	+(+)	++	+++	++	+++	+++
0	0	0	0	(+)	+	+(+)	++	+++	+++
0	0	0	0	+	++	++	++	+++	+++
++	+(+)	++	+++	+++	+++	+++	+++	+++	+++
++	+(+)	++	++	+++	+++	+++	+++	+++	+++
+(+)	++	+++	+++	+++	+++	+++	+++	+++	+++
+(+)	++	++	+++	+++	+++	+++	+++	+++	+++
++	+	++	++	+++	+++	+++	+++	+++	+++
+++	+	++	++	+++	+++	+++	+++	+++	+++
+	+	++	++	+++	+++	+++	+++	+++	+++
+	+	+	+(+)	+(+)	+(+)	+(+)	+++	+++	+++

growth, to +++ = strong growth.

and mitis, and the results should be read after 1 day's incubation. Further examinations at this temperature confirmed the earlier results, but it was found to be certain individual variations between the strains, and small variations of the temperature might give unreliable results.

No difference was demonstrated in the resistance of the 3 types against phenol or active, normal guinea-pig serum, in their viability in old cultures or in their ability to grow in deep agar cultures.

Discussion.

A distinct difference between the growth capacity of the 3 types of the diphtheria bacillus at higher temperatures than the optimum has been demonstrated. Mitis is considerably more thermo-labile than gravis, whereas intermedius takes an intermediate position. When the strains are cultivated in phosphate broth at 40° for 24 hours, gravis will grow, whereas mitis does not grow, and this is undoubtedly a method by which the types may be distinguished. Further examinations did, however, show that small variations in the temperature and individual differences between the strains might give unreliable results.

As mitis was distinctly sensitive against temperatures higher than 37°, this may possibly be the explanation of light-growing strains of this type described by some authors on tellurite media. Diphtheria cultures should therefore be incubated at 36—37°, and not above 37°.

Conclusions.

1. Gravis grows at higher temperatures than mitis, while inter-mediis takes an intermediate position.
2. Cultivation at 40° is a method by which the types can be distinguished.
3. Mitis is very sensitive to temperatures slightly higher than 37°.

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THE FERMENTING POWER OF PNEUMOCOCCI

By Erna Mørch-Lund.

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An examination of the fermenting power of the pneumococci has been undertaken in Denmark at intervals of some years. In 1913 *Christiansen* studied their fermentative effects on 22 sugars, polyvalent alcohols and glucosides, using Hiss's medium (one part horse serum to two parts distilled water). The material consisted of 38 pneumococcus strains: 23 from calves and 15 from man. All these pneumococci fermented glucose, galactose, fructose, mannose, lactose, sucrose, maltose, trehalose, raffinose, dextrin and starch. None of the strains fermented sorbitol, rhamnose, dulcitol, adonitol, mannitol, sorbose, perseitol or erythritol. Their behaviour towards inulin, cellobiose and salicin varied, and fermentations in the latter group were often late in appearing (3 to 12 days) whereas in the former they were observed within 24 hours.

In 1937 *Jürgens* tested 112 pneumococcus strains on 31 carbohydrates. The strains were equally distributed over Types 1, 2, 3 and Group X. He found that they all fermented glucose, galactose, maltose, lactose and sucrose, but that none fermented arabinose, xylose, dulcitol, sorbitol or inositol. Their behaviour as regards inulin, mannitol, melibiose and salicin varied. The latest reactions were on the fourth day; with the carbohydrates which were fermented by all the strains the reaction appeared on the first or second day. The author recommended plentiful inoculation of the fermenting tubes.

In 1944 *Langvad-Nielsen* at the State Serum Institute studied the fermenting power of 137 pneumococcus strains belonging to 64 types. The tests comprised 12 sugars dissolved in Hiss's medium (one part of ox serum and two parts of distilled water). As in the case of *Jürgens* he found that all the strains fermented glucose, galactose, lactose and sucrose, whereas none reacted with arabinose, xylose, dulcitol or inositol. Their fermenting power varied as regards inulin, mannitol, melibiose and salicin. The nonencapsulated pneumococci fermented in the same manner as the homologous, encapsulated strains.

Among publications abroad in recent years attention may be drawn to a work on pneumococcus fermentation by *Viktorow, Semzowa & Sinjuschina* (1933). The material consisted of 88 strains of Types 1, 2, 3 and Group X, which were tested against 22 sugars; the tubes were observed for five days. On the whole the results are in conformity with those of the other authors mentioned (Table 1).

Own investigations.

Since *Langvad-Nielsen's* work was concluded 9 more types of pneumococcus have been set up at the State Serum Institute (Morch 1944). With 151 strains belonging to all the 73 known types the author has again examined the question of the fermenting power of the pneumococci, with results as outlined below.

Fourteen sugars were employed: arabinose, xylose, dulcitol, inositol, sorbitol, glucose, galactose, lactose, sucrose, maltose, inulin, mannitol, salicin and aesculin. On the whole, the same sugars were used as in *Langvad-Nielsen's* work (with the exception of melibiose, which was unprocurable), and the range was supplemented by the sugars sorbitol, maltose and aesculin.

The concentration of the sugars to be fermented was $\frac{1}{2}$ % (aesculin 0.2 %). The medium was Hiss's (one part of ox serum and two parts distilled water), and the indicator was phenol-red:

Ox serum	80 c.c.
Distilled water	160 c.c.
Phenol-red	3 c.c.

The medium was steamed for about one minute before the addition of the sugars, then tubed 2 cm. depth into narrow test tubes (inner diameter 9 mm) and steamed for 10 minutes. The final pH was about 8.0.

Two drops of a young culture in serum broth were added to each of the 14 tubes and to a control tube with Hiss's medium. The tubes were shaken and incubated at 37° C. for 5 days, being inspected daily. Only a distinct change of colour (red to yellow) together with coagulation was reckoned as a positive reaction. Fermentation ended within five days and in most cases within 24 hours. The effectiveness of the inoculation was confirmed by subculture of the control tube. If the growth taken from the control tube 24 hours after the inoculation was poor, the fermentation test was repeated.

An attempt was made to employ various sugarless media for the primary inoculating culture, but the growth of the pneumococci in such media was so slight, that even the inoculation of several drops of the culture into the fermentation tubes failed to produce growth in some of them.

It was therefore decided to make use of serum broth with a low content of glucose (1 ‰). After inoculating with two drops of this culture there was in most cases a change of colour from red to orange, but never to yellow or accompanied by coagulation.

All the pneumococci fermented glucose, galactose, lactose, sucrose and maltose. With an inoculum of two drops all 151 strains fermented glucose, sucrose and maltose on the first day. With few exceptions lactose and galactose also fermented within 24 hours. One strain fermented lactose on the third day, and three fermented galactose on the 2nd, 2nd and 3rd days respectively.

None of the strains were able to ferment arabinose, xylose, dulcitol, inositol or sorbitol. Only some of them fermented inulin, mannitol, salicin or aesculin.

Inulin was fermented by 60 % of the strains, most of them on the first day (1st day: 84 %, 2nd day: 13 %). Twenty per cent of the strains were positive in mannitol, most of them on the 3rd and 4th days (1st day: 3 %, 2nd day: 20 %, 3rd day: 35 %, 4th day: 32 %, 5th day: 10 %). Salicin was fermented by 71 % of the strains, mostly on the first and second days (1st day: 44 %, 2nd day: 30 %, 3rd day: 12 %, 4th day: 8 %, 5th day: 6 %). Twenty-three per cent of the strains were positive in aesculin, 63 % on the first day, the rest within five days.

Table 1.
Fermentation Reactions of Pneumococci.

Author:	Year	No. of strains:	Fermentation		
			negative	positive	negative or positive
Christiansen	1913	38	du-so-mt	gl-ga-la-suc-ma	in sal
Viktorow et al.	1933	88	du-it-so. ar and xy weakly positive.	gl-ga-la-suc-ma	in: positive as a rule mt: 27 ⁰ / ₁₀₀ positive sal: 82 ⁰ / ₁₀₀ ,
Jürgens	1937	112	ar-xy-du-it-so	gl-ga-la-suc-ma	in: 97 ⁰ / ₁₀₀ , mt: 47 ⁰ / ₁₀₀ , sal: 59 ⁰ / ₁₀₀ ,
Langvad-Nielsen	1944	137	ar-xy-du-it	gl-ga-la-suc	in: 62 ⁰ / ₁₀₀ , mt: 34 ⁰ / ₁₀₀ , sal: 92 ⁰ / ₁₀₀ ,
Moreh	1948	150	ar-xy-du-it-so	gl-ga-la-suc-ma	in: 60 ⁰ / ₁₀₀ , mt: 20 ⁰ / ₁₀₀ , sal: 71 ⁰ / ₁₀₀ , aes: 23 ⁰ / ₁₀₀ ,

ar = arabinose, xy = xylose, du = dulcitol, it = inositol, so = sorbitol, gl = glucose,
ga = galactose, la = lactose, suc = sucrose, ma = maltose, in = inulin, mt = mannitol,
sal = salicin, aes = aesculin.

On comparing these results with those of previous authors we find agreement on the whole (see Table 1). It is agreed that all pneumococci ferment glucose, galactose, lactose, sucrose and maltose.

Viktorow et al. state that arabinose and xylose are fermented slightly, and it was in conformity with this that in the great majority of the strains I observed an ability to change the colour of the medium to orange or yellow, but never to cause coagulation, and therefore I have recorded these reactions as negative. Jürgens and Langvad-Nielsen also label their reactions with arabinose and xylose as negative. It is generally agreed that dulcitol, inositol and sorbitol are not fermented.

From his material of 38 strains Christiansen concluded that mannitol is not fermented by pneumococci, whereas in conformity with the other workers I found that a number of the strains (20 to 47 %) ferment this sugar. All the authors mentioned find that inulin is fermented only by some of the strains. Jürgens found 97 % positive, whereas Langvad-Nielsen and I found 62 % and 60 % positive respectively. Neufeld & Schnitzer (1928) state that inulin can be used in the differential diagnosis between streptococci and pneumococci, though they add that not all pneumococci are able to ferment this sugar. For salicin the authors in Table 1 give 59 to 92 % positive strains; I myself found 71 % positive.

The fermentation of aesculin was studied by me, but not by the other authors mentioned.

Winkle (1947) in his table shows that aesculin is not fermented by pneumococci. He also states that salicin is not fermented, which is contradicted by all the other authors referred to above. Mannitol is also given as negative. Winkle's statements are taken from various publications, and are not based on studies of his own. It is unfortunate that he does not mention from which works he obtained these figures.

In the course of the present investigation I went into the question of whether the fermenting power of a strain can be improved by subculture. Seven pneumococcus strains were subcultured daily in serum broth, five times in all. Three of the strains were salicin-negative before, but salicin-positive after the experiment; three more strains remained unchanged against salicin, whereas one was salicin-positive before, but salicin-negative after the subcultures. Similar changes were observed with mannitol: three strains became mannitol-positive, whereas the others did not change. One of the strains became able to ferment inulin. Strain 25 Lederle, which fermented galactose, lactose, sucrose and maltose on the 4th—5th days, could ferment them within 24 hours after subculturing. In other words, cultivation in serum broth may in certain cases increase the fermenting power of the strains. If I had taken the trouble to make a similar series of subcultures with all the tested strains before the fermentation tests, the percentages for the strains fermenting inulin, mannitol, salicin and aesculin would doubtless have been higher. My material consisted

exclusively of strains which had been stored for several years. In all probability, strains from freshly isolated material will prove to possess a better fermenting capacity than these museum strains.

In most cases, strains within the same pneumococcus type differ in their fermenting power (Table 2).

Table 2.
Fermentation Reactions of Pneumococci.
Types 1, 2, 3 and Groups 6, 10 and 19.

Pneumococcus type	strain	Aesculin	Inulin	Mannitol	Salicin
1	Neufeld	—	+ ¹	—	—
1	Neufeld R	—	+ ¹	—	—
1	Lederle	—	+ ²	—	+ ²
1	519/43	—	—	—	+ ²
1	519/43 41°×300	—	—	—	—
2	Lederle	—	—	—	—
2	M 57	—	—	—	+ ²
2	529/43	—	+ ¹	—	+ ¹
3	No. 2	—	+ ¹	—	—
3	Jersey	—	+ ¹	—	+ ²
6A	Nr. 34351	+ ¹	+ ¹	—	+ ¹
6A	Fiorelli	+ ¹	—	—	+ ¹
6A	Lederle	—	—	—	+ ¹
6B	753/39	—	—	—	+ ¹
6B	2616/39	—	—	—	—
10	No. 34355	—	—	—	—
10	6118/39	—	—	—	—
10A	Reilly	+ ²	+ ¹	—	+ ²
10A	10061/38	+ ¹	+ ¹	—	+ ¹
19	Lederle	—	—	+ ³	+ ²
19	No. 34364	+ ³	—	+ ³	+ ²
19A	977/39	—	+ ¹	—	—
19A	1777/39	—	+ ¹	—	+ ³
19B	5928/39	+ ¹	+ ¹	+ ¹	+ ²
19C	7588/40	—	—	—	+ ⁵
19C	408/41	—	—	+ ⁵	+ ²

— no fermentation.

+¹ fermentation on the first day; the number indicates the day when the reaction became positive.

The three strains of Type 1 (1 Neufeld, 1 Lederle, 1 519/43) have different fermenting power, but Pn. 1 Neufeld and the corresponding non-capsulated strain (R-form) Pn. 1 Neufeld R give the same fermentation results. After 300 subcultures in serum broth at 41 C. the strain pn. 1 519/43 had lost its power to ferment salicin, but was still able to ferment glucose, galactose, lactose, sucrose and maltose within 24 hours.

Three strains of Type 2 all ferment differently, and two strains of Type 3 have given different results. The other pneumococcus types reveal a similar behaviour. Nor is there any uniformity within the various pneumococcus groups. Only one group (Group 10) shows a conformity between the serological types (Types 10 and 10 A) and the fermenting reactions (Table 2). Whether this will hold good for a greater number of strains is open to doubt. In Table 2 I have included Groups 6 and 19 as examples of pneumococcus groups in which there is no conformity between the serological types and their fermentation reactions.

Thus on the whole I have found no agreement between the serological types and their fermenting power. Whereas the type characters of a pneumococcus depend upon the capsular antigens present, its fermenting power presumably depends upon conditions in the bacterial body.

Summary.

The author has tested 151 strains belonging to 73 pneumococcus types for their fermenting power on 14 sugars. The results are compared with those of earlier authors.

All the strains ferment glucose, galactose, lactose, sucrose and maltose. None of them ferment arabinose, xylose, dulcitol, inositol or sorbitol. Their behaviour as to inulin, mannitol, salicin and aesculin varies; 60, 20 71 and 23 % respectively of the strains ferment in these four media. Subculturing may improve the fermenting power. No correlation between serological type and the fermentation capacity could be demonstrated.

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STAINING OF HUMAN BONE MARROW AFTER DECALCIFICATION

By *Harald K. Kristensen.*

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It is generally realized that decalcification with 5 % nitric acid prevents granule staining of the cells of the bone marrow. In this respect *Custer's* announcement (1933) of a mixture of 85 % formic acid and 20 % sodium citrate was a progress. This author stated that decalcification for 6 hours usually was sufficient for a bone button of about 1 cm. in diameter. Furthermore the tissue might be left overnight without destroying the staining quality of the cells.

The above named method was used by *Harald Gormsen* in his thesis (1942). *Gormsen* wrote that this method of *Custer's* allowed him to decalcify some mm. thick plates of cancellous bone in 2—4 hours and moreover allowed good staining results, even by means of azure cosin.

Meanwhile *Lillie* (1944) still found problems in combining rapid and satisfactory decalcification and satisfactory Romanowsky staining of bone marrow. Due to this *Lillie* studied the effect of immersion for varying periods of time in various decalcifying agents on Romanowsky staining of rabbit marrow removed from the long bones. Even treatment for a short period of time with 5 % nitric acid or 5 % sulfurous acid seriously impaired marrow staining with cosin azure stains, producing a diffuse oxyphilia of nuclei as well as of cytoplasm.

As *Lillie* believed that the injury to marrow staining might be controlled by regulation of the initial pH of the fluids, series of buffered mixtures of formic acid were made.

Buffered sodium citrate formic acid mixtures with initial pH above 3.0 decalcified cortical bone slowly, but permitted good marrow staining even with several day's exposure beyond apparent decalcification.

Buffered formic acid solutions with initial pH about 2.5 decalcified more quickly, but showed tendency to impair marrow staining and were generally found no better than the 5 % aqueous formic acid.

Buffered acetic (pH 3.4—4.6) is a relatively insufficient decalcifying agent.

Trichloroacetic acid is a good decalcifying agent but on account of the high molecular weight more is required. Moreover the cost is rather high.

Nitric acid and sulfurous acid decalcify promptly but the first as promptly spoils Romanowsky staining and the second tends to do so if decalcification takes two days or more.

Decalcification investigations with the purpose to find a method which allowed galloxyanin staining of the Nissl-substance of internal ear ganglion cells made me perform some investigations of bone marrow decalcification. The primary result was that the organic acids, specially the trichloroacetic acid, were best whereas the strong and middle strong inorganic acids gave bad staining results.

Investigations on the influence of decalcification upon nucleus staining by means of galloxyanin showed that mixtures of formic acid and sodium formiate with pH 2.2—3.8 gave comparatively quick decalcification without damaging the nucleus staining.

After this it was natural to examine the influence of these mixtures upon bone marrow staining. For this purpose was employed human ribs which at once after the removal were fixed in fresh made Kaformacet (5 % potassium bichromate, 425 cc — 40 % formalin, 50 cc. — 96 % glacial acetic acid, 25 cc. — distilled water, 500 cc.). After fixation for 24 hours and washing in tap water for 24 hours 6—8 mm. thick pieces were sawn out.

These rib pieces were put into ten various mixtures of 1 N, 2 N, 4 N and 8 N formic and 1 N sodium formiate (highest pH 3.8, lowest pH 2.2). The rib pieces were suspended in the acid mixture by means of a thread in order to make the diffusion of the calcium salts easier.

The following scheme shows the decalcification times:

No	pH	Time
1	3.8	about 5 days
2	3.5	» 3½ day
3	3.2	» 3 days
4	3.35	» »
5	3.25	» »
6	2.85	» 2 days
7	3.0	» 1½ day
8	2.7	» 30 hours
9	2.3	» 24 »
10	2.2	» 18 »

The bone pieces were washed in running water for 24 hours, transferred to increasing strengths of alcohol, to xylol and paraffin (melting point 56° C.).

The blocks were cut in sections of about seven micra in thickness, and mounted on slides.

After removal of paraffin staining took place in:

Bohmers hematoxylin

Einarsons gallocyenin

Maximows buffered azure eosin.

Staining with hematoxylin showed the nuclei sharp with chromatic of blue or black blue colour. The mature red blood cells were practically unstained. No difference could be seen in preparations from various acid mixtures.

Calloccyanin staining showed the nuclei as sharp and nearly as intense as after the hematoxylin staining. The red blood cells were completely unstained. Neither here any difference was seen in the various preparations.

The azure eosin staining was a slight modification of the Maximow method. The following solutions were used:

Solution I: Eosin 1 gram

Distilled water 1000 cc.

Solution II: Azure 1 gram

Distilled water 1000 cc.

Just before the use a mixture was made of:

Solution I, 10 cc. + 100 cc. buffered water and
solution II, 15 cc. + 100 cc. buffered water*).

The following procedure was employed:

- 1) Stain in the above mentioned stain mixture for 24—48 hours.
- 2) Quick rinsing in buffered water.
- 3) Half a minute in acetic acid water (a few drops of acetic acid to 200 cc. distilled water).
- 4) 96 %, 100 %, 100 % alcohol, half a minute in each.
- 5) Toluol, 3 changes and at least 5 minutes in each.

In the acetic acid water red stain goes off, in 96 % alcohol blue stain goes off. Control under the microscope.

When this schedule is followed uniform and excellent staining results are found and it makes no difference if the preparations are decalcified in formic acid sodium formiate mixtures of various acidity. Therefore the most fast decalcifying mixture (equal parts 8 N formic acid and 1 N sodium formiate) is recommended.

In the preparations all shades between brilliant red and blue are represented.

The staining and the sharpness of mature and immature cell forms was practically as successful as in fine smears. Specially the granules of the eosinophile granulocytes were found distinct and brilliant red.

Overdecalcification made no influence upon the stain quality.

*) Buffered water made of 10 cc. buffer solution (equal parts of primary and secondary potassium phosphate solutions) and 100 cc. distilled water.

Summary.

During some decalcification investigations a decalcification method based upon the decalcifying effect of a mixture of formic acid and sodium formiate was brought forward. The principle of the method is comparatively rapid decalcification in spite of rather high pH. The method allows excellent staining with hematoxylin, gallocyanin and buffered azure eosin.

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A NEW SALMONELLA TYPE (*S. TINDA*) FROM THE BELGIAN CONGO

By F. Kauffmann and P. G. Janssens.

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This culture was isolated in Bambu in 1946 from the water of a spring in Camp Tinda and sent to Copenhagen for diagnosis by Dr. Courtois, Blukwa, Congo Belge.

The biochemical behaviour of this new type is as follows: No fermentation of adonitol, lactose, salicin and sucrose. No production of indole and no liquefaction of gelatin. Rapid fermentation of arabinose, duleitol, glucose (with gas), inositol, maltose, mannitol (with gas), rhamnose, sorbitol, trehalose, and xylose. Positive reaction in Stern's glycerolfuchsin broth, and formation of H_2S . Prompt growth on Simmons' agar containing glucose and sodium citrate. Positive reaction in d-tartrate, l-tartrate, mucate, and sodium-citrate after 1 day; negative reaction in i-tartrate after 14 days.

Serologically the type has the formula I, IV, XXVII, XII: a: e,n,z₁₅, i.e. a type with α - β phase-variation.

Cross-absorptions showed that the O antigen I, IV, XXVII, XII is identical with the O antigen of *S. bredeney*, the H antigen α with the H antigen of *S. paratyphi* A, and the H antigen e,n,z₁₅ with the H antigen of *S. potsdam* phase 2.

Summary.

The authors describe a new *Salmonella* type from the Belgian Congo: *S. tinda* = I, IV, XXVII, XII: a: e,n,z₁₅.

LACTOBACILLUS BULGARICUS AS TEST ORGANISM IN PENICILLIN AND STREPTOMYCIN ASSAYS

By G. Tunevall.

(Received for publication April 7th, 1949.)

Staphylococci, streptococci and klebsiellae most commonly used as test organisms in penicillin and streptomycin assays need for their development at least 18 hours. Especially streptomycin treatment ought to be rapidly adjusted according to frequent determinations of the streptomycin content of blood or other body fluids in order to keep an adequate concentration. So, a rapid method for assays making possible to avoid one day's delay should mean an important help in the control of antibiotic treatment.

As described in 1947 by Sanchez & Lamensans (1) *Lactobacillus bulgaricus* in skimmed milk at 45° C develops in 2½ hours, in the same time bringing about coagulation of the medium. These activities are inhibited by small amounts of commonly used antibiotics. In 1948 the same authors (2) designed a method for rapid assays of penicillin, streptomycin and tyrothricine by use of *L. bulgaricus*. A simple modification of this method has been used in our laboratory for determination of the penicillin and streptomycin content of blood and other body fluids.

As test organism for penicillin a strain of *L. bulgaricus* is used, isolated from commercial Yoghurt milk. Strains obtained from several type collections presented less favorable properties than the commercial strain which is very rapidly growing, brings about a complete coagulation and is sensitive to 0.007 I. U. penicillin per ml of the medium. This strain however is fairly resistant to streptomycin.

In streptomycin assays a strain is used which was kindly put to my disposal by Drs Sanchez and Lamensans. The strain is sensible to 0.5 W. U. streptomycin per ml medium but less sensible to penicillin than the commercial strain.

The test strains are maintained by adding 0.1 ml culture to 10 ml skimmed milk (autoclaved at 115° C for 5 minutes) and incubating the mixture at 45° C until coagulation is complete (2½—3½ hours).

Table I.
PENICILLIN
Fluid to be tested.

I. U. pen. per ml	Dilution		1/3	1/6	1/12	1/24	1/48	1/96	1/192	1/384
	Tube nr		1	2	3	4	5	6	7	8
Standard.	0,020	1	0,04 — 0,06 — 0,08 — 0,12 — 0,17 — 0,24 — 0,34 — 0,48 — 0,68 — 0,96 — 1,3 — 1,9 — 2,7 — 3,8 — 5,4 — 7,7							
	0,013	2	0,03 — 0,04 — 0,06 — 0,08 — 0,11 — 0,16 — 0,23 — 0,32 — 0,45 — 0,64 — 0,91 — 1,3 — 1,8 — 2,6 — 3,6 — 5,1							
		3	0,02 — 0,03 — 0,04 — 0,05 — 0,08 — 0,11 — 0,15 — 0,21 — 0,30 — 0,43 — 0,60 — 0,85 — 1,2 — 1,7 — 2,4 — 3,4							
	0,006	4	0,01 — 0,02 — 0,03 — 0,04 — 0,05 — 0,07 — 0,10 — 0,14 — 0,20 — 0,28 — 0,40 — 0,57 — 0,80 — 1,1 — 1,6 — 2,3							
		5	0,01 — 0,01 — 0,02 — 0,02 — 0,03 — 0,05 — 0,07 — 0,09 — 0,13 — 0,19 — 0,27 — 0,38 — 0,54 — 0,76 — 1,1 — 1,5							

Table 2.

STREPTOMYCIN

Fluid to be tested.

W.U. strim per ml	Dilution		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	Tube nr		1	2	3	4	5	6	7	8
1,6	1	2,2—3,3—4,5—	6,5—9,0—	13—18—	26—36—	51—71—	100—140—	200—290—	410	
		1,8 2,6 3,6 5,2	7,2 10 14 21	28 42 55 83	110 170 230 330					
1,1	2	1,5—2,1—3,0—4,3—	5,9—8,6—	12—17—	24—34—	48—68—	95—140—	190—270		
		1,2 1,7 2,4 3,4	4,9 6,9 10 14	19 28 38 55	75 110 150 220					
0,7	3	1,0—1,4—2,0—2,8—	4,0—5,7—	8,0—11—	16—23—	32—46—	64—91—	130—180		
		0,8 1,2 1,6 2,3	3,2 4,6 6,3 9,2	12 18 25 37	50 74 100 150					
0,5	4	0,7—1,0—1,3—1,9—	2,6—3,8—	5,2—7,6—	10—15—	21—30—	42—61—	85—120		
		0,5 0,8 1,1 1,5	2,0 3,1 4,3 6,1	8,5 12 17 24	35 49 71 98					
0,3	5	0,4—0,6—0,8—1,3—	1,5—2,5—	3,6—5,0—	7,1—10—	14—20—	28—40—	56—81		
		0,3 0,5 0,7 1,0	1,3 2,0 2,9 4,0	5,6 8,1 12 16	23 32 46 65					
0,2	6	0,3—0,4—0,6—0,8—	1,2—1,6—	2,3—3,3—	4,7—6,6—	9,4—13—	19—27—	38—54		

Standard.

The cultures may be stored at + 4° C for one week but to make sure of rapid growth daily recultivations are to recommend. For the assays a mixture of 2.5 ml culture and 100 ml skimmed milk is made up and shaken with glass beads to ensure a homogenous mixture. All procedures may be performed without any consideration to sterility.

Titration.

Penicillin:

<i>Standard series:</i>	Tube nr.	1	2	3	4	5	6
Penicillin standard 0,06 I. U./ml		0,5	1,0	—	—	—	—
Saline solution		—	0,5	0,5	0,5	0,5	0,5
1 ml is passed from 2 to 3, from 3 to 4 and so on.							
Culture-milk mixture		1,0	1,0	1,0	1,0	1,0	1,0
Final penicillin content I. U./ml.		0,020	0,013	0,009	0,006	0,004	0,003

<i>Test series:</i>	Tube nr.	1	2	3	4	5	6	7	8
Fluid to be tested		0,5	0,5	—	—	—	—	—	—
Saline solution		—	0,5	0,5	0,5	0,5	0,5	0,5	0,5
0,5 ml is passed from 2 to 3, from 3 to 4 and so on.									
Culture-milk mixture		1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0
Final dilution		1/3	1/6	1/12	1/24	1/48	1/96	1/192	1/384

Incubation in 45° C water bath is performed until coagulation is complete in a control tube without penicillin and then for additional 15 minutes (about 2½ hours).

Reading: Complete coagulation is characterized by the milk being converted into a solid coagulate from which a small amount of clear supernatant is separated. If the last tube not completely coagulated presents solid or semisolid milk but no supernatant this tube is taken as representing the end point. If this tube is completely or partially fluid, the end point is considered to lie between that tube and the first one presenting complete coagulation.

Calculation is readily made by use of table 1.

Streptomycin:

<i>Standard series:</i>	Tube nr.	1	2	3	4	5	6
Streptomycin standard 3,2 W. U./ml. ...		0,5	1,0	—	—	—	—
Saline solution		—	0,5	0,5	0,5	0,5	0,5
1 ml is passed from 2 to 3, from 3 to 4 and so on.							
Culture-milk mixture		0,5	0,5	0,5	0,5	0,5	0,5
Final streptomycin content W. U./ml		1,6	1,1	0,7	0,5	0,3	0,2

<i>Test series:</i>	Tube nr.	1	2	3	4	5	6	7	8
Fluid to be tested		0,5	0,5	—	—	—	—	—	—
Saline solution		—	0,5	0,5	0,5	0,5	0,5	0,5	0,5
0,5 ml is passed from 2 to 3, from 3 to 4 and so on.									
Culture-milk mixture		0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Final dilution		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256

Incubating and reading as for penicillin.

Calculation by use of table 2.

The accuracy of the method.

Standard solutions containing 0.03, 0.09 and 0.27 I. U. penicillin per ml were prepared in saline solution and in normal human serum. On three consecutive days 3 assays were made daily of each saline standard and 6 assays of the serum standards. In all 81 assays were performed. If the known penicillin content of the standards are put 100, the results were as follows:

Saline standards	96 \pm 14.6
Serum standards	107 \pm 21.2
All standards	104 \pm 19.3

The mean values do not differ significantly, nor do the standard errors. So, no inhibitory effect of human serum could be demonstrated.

In the same way determinations were performed of streptomycin standards containing 1.5, 4.5 and 13.5 W. U./ml. The results were the following:

Saline standards	97 \pm 12.8
Serum standards	107 \pm 16.8
All standards	105 \pm 15.1

These mean values and standard errors indicate no effect of the serum.

Further, serum from 33 patients receiving penicillin were titrated parallelly by use of *Fleming's* method (3) with a staphylococcus (strain 209 p) as test organism and by the *L. bulgaricus* method. If the values of the Fleming method are put 100, the distribution of the *L. bulgaricus* values is represented by the figure 103 ± 25.1 . The difference between the values of the two methods is insignificant.

Summary:

By use of *Laetobacillus bulgaricus* penicillin and streptomycin assays can be performed in about 3 hours with sufficient accuracy for clinical purposes. Infected or contaminated body fluids may be used. A simple method for the assays is described and the errors of the method are calculated.

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BACTERIAL MUTATION WITH CAFFEINE

By J. Ørskov.

(Received for publication April 19th, 1949.)

The minor investigation to be described in the following was the outcome of impulses received partly from works by *Niels Fries & Bengt Kihlman*¹ and *Bengt Kihlman & Albert Levan*², and partly from earlier works on the remarkably selective, morphogenetic influence of caffeine on bacteria of certain kinds³. *Gamaliya*, by the way, seems to have been the first to show (about the beginning of the present century) that in nutritive media caffeine has this curious effect on the morphology of these bacteria. My own previous experience had shown that, when present in suitable concentrations, caffeine has a strongly inhibitory action upon the ability of bacilli to divide, especially gram-negative species, causing them to grow out into long threads, often with very remarkable spindle-shaped and globular swellings. As long as these bizarre elements are still relatively young and their protoplasm is homogenous, they are mostly capable, when transplanted to other media without caffeine, of growing and producing fairly quickly what we call normal growth forms again. When allowed to remain on caffeine medium for some time the protoplasm undergoes changes, becomes granular and vacuolized, and such forms, to an increasing degree according to the intensity of the changes, lose their ability to grow on fresh media.

The bacterium selected for these experiments, one taken from a soil sample, is a non-motile, gram-negative, capsulated bacillus which when growing normally often lies in pairs, for which reason one is tempted to call it a diplobacillus, and ferments all the usual sugars and is extremely moderate in its nutrition requirements, for example growing to visible colonies on aqueous agar without the addition of extra nutritives. It grows well at both 37° and room temperature, at the latter of which most of the experiments were made. In the presence of glucose and saccharose the colonies become very slimy, caused partly by the extra-cellular formation of polysaccharides. The mutants or variants described below were never observed under normal conditions of growth.

All morphological examinations were made by direct agar microscopy⁴: a cube of the agar is cut out, placed upon a slide, whereafter examination can be made at all magnifications commencing with a low-power dry lens, ending with oil immersion after a cover-slip has been placed over the preparation. One works most conveniently with large slides and cover-slips. Ideal illumination is of importance, and in this excellent working conditions can be secured with the Leitz Ortholux; all examinations were made with that microscope. When cultivating from very small colonies not clearly distinguishable with the naked eye, seeding was performed with the aid of a primitive micromanipulator, with which the seeding procedure can be followed direct under the microscope.

If this microorganism is cultivated in Petri dishes on the surface of e. g. an agar with 3 % broth and $\frac{1}{2}$ % caffeine, *all* rods grow out into long, twisted threads without lines of division, and very soon there appear the aforesaid curious swellings and globules; after a few days' growth the degeneration of the protoplasm begins and growth ceases. Fig. 1.

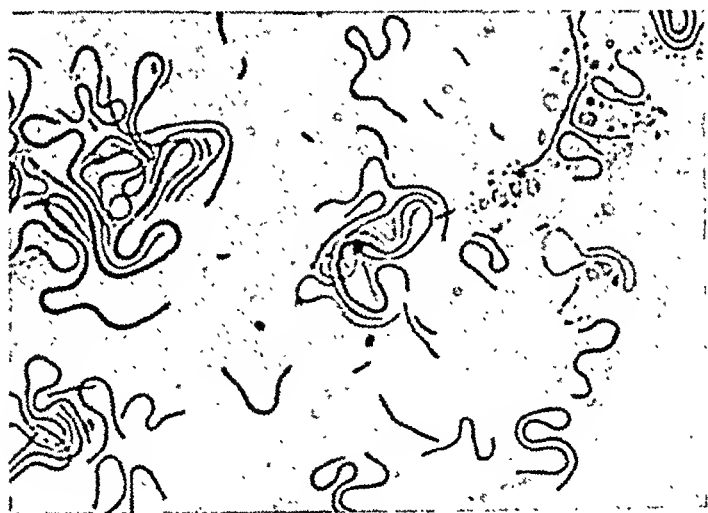


Fig. 1.

Diplobacillus. 3 days growth, room temperature.

3 % broth and $\frac{1}{2}$ % caffeine.

Oil Apochromat 60 (0.85). Ocular 10 × B.

Then, in the best of cases, after a few days' growth there appear scattered small colonies consisting of bacteria that are capable of dividing in the normal manner, except that the process is exceedingly slow. Sometimes it takes many days for the colonies to become so large that they can just be discerned with the naked eye in the most favourable illumination. On subculturing from these dwarf colonies to media without caffeine one obtains a pure culture of bacteria having a perfectly normal appearance, but distinguished by growing infinitely

slower than the »mother« bacillus; even on what we would call a good broth agar, on which one of the original bacteria grows into a large, rich colony in the course of 24 hours, in the case of the variant it is days before one can just see the colony grown from a single bacterium. Evidently the variant has lost one or more properties while on the caffeine medium. At the same time it has acquired an ability to divide in a regular, if slow, manner on caffeine medium, an ability which it retains but without any noteworthy increase in its resistance to caffeine in stronger concentrations. Parenthetically I may add that the addition of e. g. glucose causes no great increase in the velocity of division.

But if the small, slowly growing mutant is transplanted e. g. to a 30 % ascites agar or 10 % blood agar, it will grow almost like the »mother« bacillus. In other word, ascites or blood must contain substances that fill the »gap« caused by the mutation.

It was an obvious idea to try the effect of adding amino acids to a medium, such as a good broth agar, and a primitive test with the available amino acids showed that some of them, especially cysteine and cystine, could practically even up the difference in growth between the original bacillus and the mutant.

The experiment was a rough one, a broth agar being seeded diffusely with the mutant, whereafter a small drop of amino acid solution (with cysteine and cystine usually saturated) was sprinkled upon the culture. Later on a similar addition of cysteine was tested on aqueous agar (Fig. 2 and 3), and here again, with a centrally placed

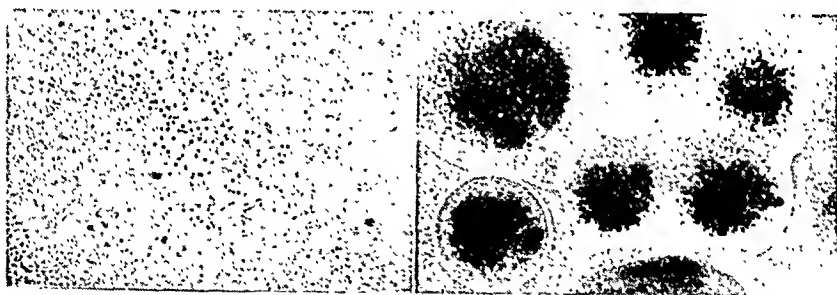


Fig. 2.

Fig. 3.

The slowly growing mutant on aqueous agar without and with cystein.

Five days' growth at room temperature. Zeiss A 8—0.2. Ocul. 10 × B.

drop diluted 1 : 5, there was growth just as vigorous as the mother bacillus can show without cysteine (which, be it noted, had no stimulating influence on the mother bacillus). This cysteine effect, which naturally was most pronounced at the centre of the dish where the drop was placed and the concentration highest, in the course of a couple of days at room temperature had very obviously reached right out to the edge of the Petri dish 5—6 cm. from the centre, though the concentration there must have been extremely low. Subculturing

from both ascites agar and agar with cysteine constantly gives pure cultures of the slow mutant within the first few days.

The table shows the effect obtained with the various amino acids. Naturally, the strongly acid compounds were strongly inhibitive where the concentration was highest, but they had an easily distinguishable effect outside the sharply delimited zone of inhibition.

Table.

1. dl-Alanine	0	
2. l-Arginine-HCL	+	
3. l-Aspartic acid	0	(centrally inhibitive)
4. l-Cysteine-HCL	+++	(centrally strongly inhibitive over whole drop)
5. l-Cystine	+++	(no central inhibition)
6. dl-3,4 Dihydroxy-phenylalanine	0	
7. l-Glutamic acid	+	(ccentral inhibition)
8. Glycine	0	
9. l-Histidine-HCL	0	
10. l-Hydroxyproline	0	
11. dl-Isoleucine	(+)	(slight central inhibition)
12. l-Leucine	0	
13. dl-Lysine-HCL	0	
14. dl-Methionine	0	
15. dl-Norleucine	0	
16. dl-Phenylalanine	0	
17. l-Proline	0	
18. dl-Serine	0	
19. dl-Threonine	((+))	
20. l-Tryptophane	((+))	
21. l-Tyrosine	0	
22. dl-Valine	0	

It remains to say that the mutant possesses a distinct tendency to mutate back to growth forms which in their colonies are morphologically like the initial strain. These mutants arise on all media most easily seen in media that are »insufficient« for the mutant, readily recognizable for instance on broth agar, and are divisible into two sharply separate types: one, the most common, which grows at a rate that lies between the original and the slow and whose colonies never attain to the size of those of the mother bacillus, and a rarer type which is indistinguishable from the latter. Cysteine also has a certain influence on the growth of the »intermediate« type. When suitable, supplementary growth conditions are provided for the slow mutant, it ferments a number of sugars in exactly the same way as the »intermediate« form and the mother bacillus. For the purpose of obtaining clearer evidence if possible of the effect of pure cysteine on the growth of the mutant I had a fluid synthetic medium prepared as described by *Henderson & Snell*⁵, except with the omission of the vitamins, purines and amino acids. The medium otherwise was composed as follows, with and without l-cysteine.

Synthetic medium A.

Component	Quantity per 10 ml. tube
	mg.
Glucose	200
Sodium citrate	200
Sodium acetate	10
NH ₄ Cl	30
K ₂ HPO ₄	50
MgSO ₄ · 7H ₂ O	8
FeSO ₄ · 7H ₂ O	0.4
NaCl	0.4
MnSO ₄ · 7H ₂ O	1.6
The medium was adjusted to pH 6.8.	

Synthetic medium B.

Like Medium A but plus 2 mg. l-cysteine per tube.

The result was that the mutant grew splendidly at room temperature when the medium contained cysteine, and not at all without it. (My thanks to Mr. Vagn Møller for preparing this medium). On the other hand, the mother bacterium grew well on the medium without cysteine, whereas the »intermediate« form grew fairly well, but relatively slowly.

Summary.

When a gram-negative, capsulate diplobacillus cultivated from a soil sample and only very little exacting as to nutrition was subcultured on a medium containing caffeine, its division was inhibited, as demonstrated previously in the case of many gram-negative bacteria, and the bacterium grew out into long threads presenting the familiar involutive picture. Mutants arose under suitable experimental conditions, able quite slowly to divide and form small colonies. After pure cultivation of these mutants it was found that they had become dispossessed of part of the growth powers of the mother bacillus, manifested in extremely slow growth even on relatively good media such as broth agar. Cultivation on ascites agar or blood agar abolished the difference almost completely. Rescuing on poorer media was followed by renewed slow growth.

The addition of certain amino acids, more particularly cysteines, almost completely evened out the growth difference between the initial bacterium and the mutant. On a purely synthetic medium with and without cysteine there was growth only in the presence of the cysteine.

The original bacterium is thus able to synthesize all the necessary amino acids and growth factors from simple inorganic substances,

glucose, acetic acid and citric acid while the mutant has lost the ability to synthesize cysteine.

There was a distinct inclination to mutate back to the original form, manifested morphologically most frequently in an »intermediate« form of colony, less often to a form in which the colony was indistinguishable from the original.

There was no difference between the mutants and the mother form as regards the fermentation of the sugars tested.

Lately my attention has been drawn to a work by J. O. Lampen, R. R. Roepke & M. J. Jones⁶ who have examined mutants of coli bacilli caused by X-ray showing some resemblance to my caffeine mutant not being able to synthesize cysteine. The most defective mutants required the addition of homocysteine or methionine, but with these they would all grow.

We have examined our mutant, the intermediate back-mutant and the back-mutant not to tell from the original bacillus which also was tested using the basal medium plus respectively Na_2SO_3 , Na_2S , $\text{Na}_2\text{S}_2\text{O}_3$, HSCH_2COOH , $\text{HSCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ = l-cysteine, $(\text{S}(\text{CH}_2)_2\text{CH}(\text{NH}_2)\text{COOH})_2$ = dl-homocystine, $\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ = dl-methionine and found that the mutant and the »intermediate« back-mutant could only grow on medium containing l-cysteine while the back-mutant resembling the original strain just as this grew fine on the basal medium without any addition.

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ON THE SO-CALLED DOUBLE GRANULAR SYSTEM IN THE CYTOPLASM OF THE EOSINOPHILIC LEUCOCYTE

By Olavi Eränkö.

(Received for publication April 19th, 1949.)

It has long been known that the cytoplasm of the eosinophilic leucocyte contains »coarse, highly refringent, specific granules«¹. In 1865, *Schultze*² described two kinds of granules in the cytoplasm of living leucocytes, one being more, *the other less* refractive than the surrounding medium. Recently, *Wallgren* has revived and extended this view. He has claimed that the pale and the dark granules are to be found, not only in the cytoplasm of leucocytes³, but also in the cytoplasm^{5, 8} and in the nucleus^{6, 8} of various blood, bone marrow, and tissue cells as well as in the Golgi apparatus of some secreting cells^{7, 8}. *Wallgren* points out »the stubbornness with which the pale granules and the double granular substance has defied scientific research« and writes further: »They (the granules) are evidently a characteristic trait of the living substance in human cells and on this account great attention is due to them«. *Saltzman*², in his studies for the M. D. degree, closely associated with *Wallgren's* findings, »corroborated without difficulty« the presence of the double granular system.

From the point of view of the cytologist, *Wallgren's* opinions seemed to be of interest. Therefore, a study was made of the nature of the granules in the eosinophilic leucocyte. For in this kind of cell, »the mentioned drops are particularly large and thus more accessible for observation than in the other cell forms«⁵. This paper presents the results of the re-examination.

Material and Methods.

The blood was obtained from a number of healthy human subjects. A small drop from the finger tip was allowed to spread between the slide and the cover slip as a thin layer. Vital preparations thus made

were examined immediately by transmitted light. The heating of the microscope stage was found unnecessary because the characteristic movements of the leucocytes were continued long enough at room temperature. Some preparations were stained vitally with brilliant cresyl blue (Grübler).

The Zeiss optical system was: an apochromatic oil immersion objective 120 x, N. A. 1.30, compensation oculars 15 x or 20 x, binocular tube 1.5 x or 2.5 x, and an aplanatic condenser N. A. 1.40. With this system the resolving power of the microscope is about 0.21 micra.*) As a source of light Zeiss's pointolite or low voltage lamp with condenser were used.

In addition, some model experiments were carried out with oil-water emulsions and with glass balls surrounded by fluids of different refractive indices. In the latter case the balls were examined by means of a camera with an opaque glass.

Observations.

If oil drops in water are observed under the microscope the image seen depends on the position of the microscope tube. When this is raised the center of a drop becomes luminous but if the tube is lowered the drop seems dark. On the other hand, water drops surrounded by cedar oil are conversely dark with the raised position and centrally luminous with the low position of the tube. These circumstances are clearly demonstrated by *Wallgren's* figures (figs. 1 and 2 in his paper⁹). The model experiments with glass balls in different media showed further that, just as one would theoretically suppose, quantitative changes in the refractive index of the fluid influence the picture. The first-mentioned phenomenon, used by *Wallgren*⁹ to prove the existence of *refrangent* granules in the eosinophilic leucocyte, seemed, however, to be accurate enough to decide qualitatively whether granules in some medium are more or less refractive than it.

Wallgren^{5, 8} suggests that the double granular system is clearly visible in the living eosinophilic leucocyte when examined in transmitted light without changing the position of the microscope tube. By this means the cytoplasm seems, indeed, to be filled with luminous and dark granules in continuous movement. According to *Wallgren* the two kinds of granules distinctly differ from each other in consistence and in changes of shape *etc.* This is one of the principal arguments presented by him for the existence of the double granular system. The present author has not been able to confirm the differences. — In his optical system, *Wallgren*⁵ had an apochromatic objective with a numerical aperture of 0.85, and a filter of yellow or orange color, thus absorbing light rays of the shortest wave-lengths in

*) $H = 0.61 \times \lambda / N. A., \lambda \sim 0.5 \text{ micra.}$

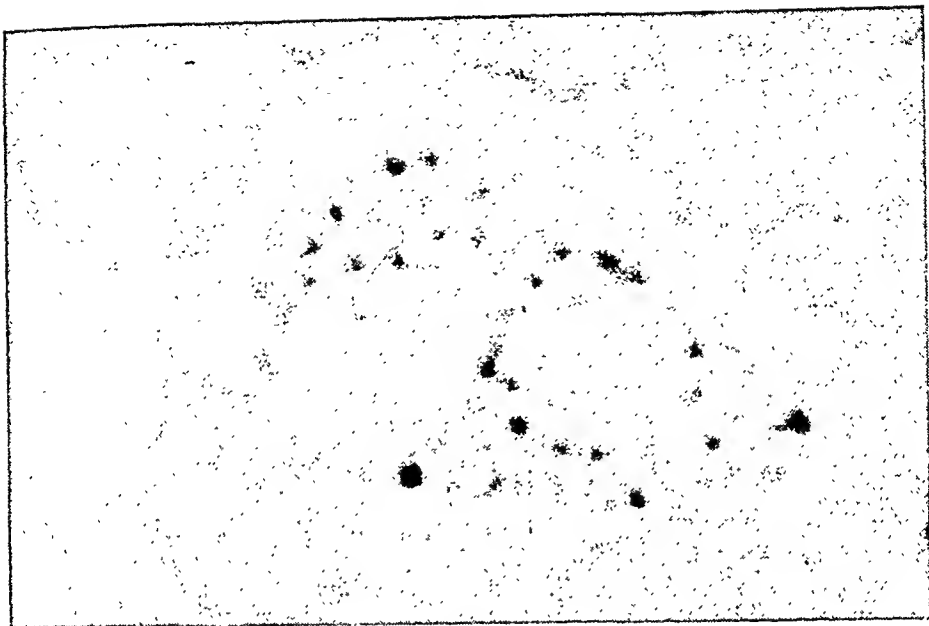


Fig. 1.

An eosinophilic leucocyte from an unstained vital preparation. Unretouched photograph taken with low position of the microscope tube.

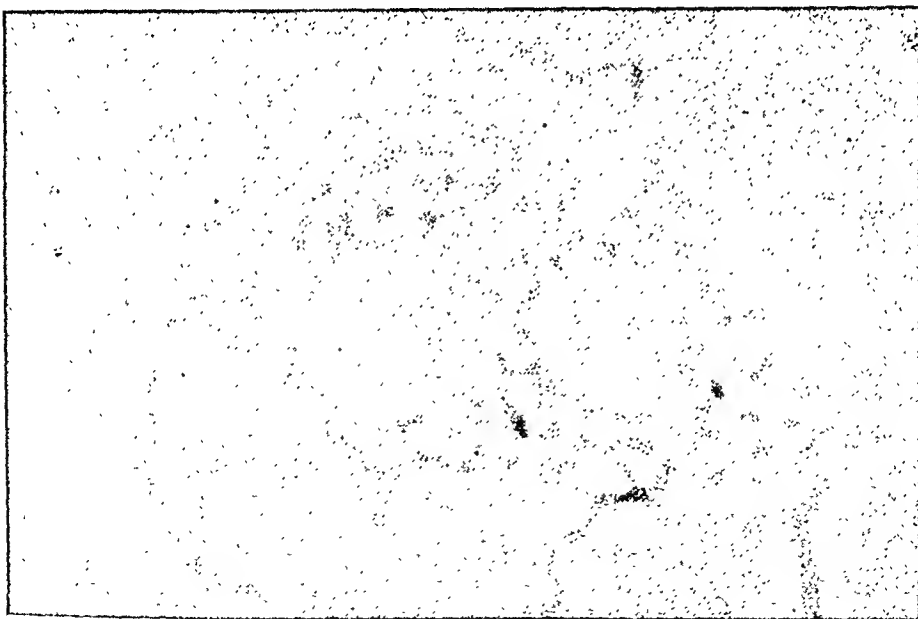


Fig. 2.

The same cell after heighening the tube. The granules, which are dark surrounded by a luminous zone in the figure 1, seem luminous with a dark zone in the figure 2. The same phenomenon is distinctly visible in the erythrocytes, these being more refractile than the surrounding medium, also. Optical system: Zeiss's carbon-arch lamp with a Cu SO_4 -filter, aplanatic immersion condenser N. A. 1.40, achromatic objective N. A. 1.25, $100\times$ (Reichert), 'Orthoskop' oculare $28\times$ (Zeiss), Contax, Agfa 'Isopan ISS' film. Magnification about $3000\times$.

the visible spectrum. We may, therefore, suppose that the resolving power of his optical system was about 0.42 micra ($= 0.6 \times 0.6/0.85$). The diameter of the largest, »dark« granules is only twice this magnitude order⁹. Thus, no very definite conclusions may be drawn regarding the shape of the granules.

The problem in question is best studied in a living cell, either stained or unstained, which has spread itself to such a thin film that only one layer of granules is visible, at least in a part of the cytoplasm. When single granules are examined it will be found that every one behaves optically like drops refracting more light than the surrounding fluid. *All the granules have a luminous center with the raised position and they are centrally dark with the low position of the tube while, on the contrary, none of them shows the reverse phenomenon as would be expected if there were granules with a lower refractive index than the surrounding medium.* In an individual granule, furthermore, the black spot will always be seen exactly below the pale one (not at the side of it as claimed by Wallgren^{5, 8}). This is true, however, with correctly centered illumination only. If one examines the granules in oblique illumination they apparently change their position when the tube is moved. If the granules are in layers above each other, some of them may seem pale and some of them dark, depending on their position below or above the optic plane of the microscope. In addition, the lower layers change the direction of and refract the light rays before these reach the uppermost layer of granules. Consequently, very complicated optical phenomena are produced, not interpretable with the simple methods used here. Wallgren's above-cited observations have been made on cells with several layers of granules, however.

Lastly some preliminary observations may be mentioned. In a phase contrast microscope the granules of the eosinophilic leucocyte are dark and no other kinds of granules are visible. Regarding the nature of the granules it is of interest that, in unstained blood smears shortly fixed with heat, refringent granules are still visible after treatment with xylene, which dissolves the sudan-positive lipids.

Conclusion.

In the light of the above-presented facts, which may easily be confirmed by everyone who has a microscope with an immersion objective, it is evident that all the visible granules in the cytoplasm of a living eosinophilic leucocyte are more refringent than the surrounding medium. *Thus no double granular system has been found in the object claimed to be most suitable for this kind of study.* The existence of such a system in other cells, having smaller granules, seems therefore doubtful.

Summary.

The cytoplasmic granules in the eosinophilic leucocyte have been examined in vital preparations. In the living cell, they are all highly refringent. The »double granular system«, recently defended by Wallgren, seems to be an optical illusion.*)

Acknowledgement.

The author wishes to express his gratitudes to Professor Ivar Wallgren for his kindness in demonstrating the leucocytes with his own optical system.

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*) Since the writing of this paper, other cells of human peripheral blood as well as some fresh tissue cells of rat have been examined by means of direct, phase contrast, and dark field microscopes. No double granular system could be observed.

ON THE DOUBLE DROP SYSTEM IN THE LIVING CELL

By Ivar Wallgren.

(Received for publication May 12th, 1949.)

I have been given the opportunity of expressing my views with regard to an article in this issue, written by O. Eränkö.

First, a correction: *Eränkö* gives an incorrect description of my optical method, evidently owing to a misunderstanding. In transmitted light I have used Zeiss' apochromatic objective H. I. 90 n.A. 1.3 K 15 x or K. 20 x. The majority of the examinations were done without a stop filter. My optics are thus not inferior to those of *Eränkö*.

In an eosinophil cell *Eränkö* observes, in transmitted light, both luminous and dark drops in the cytoplasm. »All the granules have a luminous center with the raised position and they are dark with the low position of the tube while, on the contrary, none of them shows the reverse phenomenon as would be expected if there were granules with a lower refractive index than the surrounding medium«.

Eränkö bases his statement on *Ivar Wallgren's* investigation with drops of water in oil and drops of oil in water. *Eränkö* does not take into consideration, however, that a drop, with almost similar index of refraction as the surrounding medium, gives only a very slight light-effect above or below the drop, in the microscope, depending on whether the index of refraction in the surrounding medium is lower or higher than in the drop itself. Thus, *Eränkö's* observation, quoted above, does not exclude the possibility of there being two drops, close together, with different indexes of refraction.

Eränkö's investigation concerns principally the eosinophile leucocyte in transmitted light. He thus used the same technique as *Max Schultze* did, more than 80 years ago. This eminent investigator, one of the most prominent personages in the field of biological research in the middle of the past century, says that he has definitely (»unzweifelhaft«) seen two different kinds of granules in the leucocyte. It is hardly probably that such an experienced investigator would have

fallen the victim of an optic illusion, possibly caused by an error in centering of the microscope. *Schultze* examined the living cells on a warm stage — and *Eränkö* did not. It is true that a leucocyte moves in room temperature as well, but the movements of the drops are more lively in heat. Hence it is not surprising that some details in the manner of movements of drops has escaped *Eränkö's* eye.

When the dark drops are shifted rapidly in the cytoplasm they may, for instance, be observed to take on a longitudinal, oval shape, the length becoming three times greater than the breadth. The pale drops may become slightly elongated at the same time. The majority of the pale drops remain round, however. Cells, in which the dark drops constantly enter into contact with one another, by means of thread-like bridges, are observed. The pale drops are directly united, and later they split up into smaller drops. The viscosity of the dark drops seems to be less than that of the pale ones. All these details, which favour the presence of two different kinds of drops, have not been observed by *Eränkö*. In my opinion this does not entitle him to assume that more experienced investigators have not either been able to observe these phenomena.

Eränkö imagines that black and white are different optic expressions for the same drop, and every white spot is caused by a dark drop being situated further down. If a living eosinophil cell which has spread into a very thin layer on the slide is, examined, it is found, when the tube is adjusted to the most basal dark drops, that small pale drops become visible when the tube is raised just so much as to allow the basal dark drops to remain visible all the time. In this case there are no dark drops still farther down, and yet the pale drops are observable. Thus, *Eränkö's* conception is not supported.

In a granulocyte, vitally stained with brilliant cresyl-blue, the dark drops become blue, but the pale drops remain unstained. Also this circumstance suggests that there are actually two different kinds of drops.

The dark-ground microscope is a valuable adjuvant towards clarification of the question discussed, because a drop is luminous or optically void in dark-ground illumination, and no light-effect arises above or below the drop, as it does in transmitted light, or in the phase contrast microscope. In a great number of living cells from blood or bone marrow, small, slightly luminous drops are visible in the darkfield, and small dark gaps are easily discerned in their adjacency. The characteristic thread-like bridges between the gaps are also visible. Their identity is thus established. The dark drops appear in this humble manner in the darkfield. *Eränkö* has not paid attention to these observations.

In a stained smear the neutrophil granules in a leucocyte are observed. If the same specimen is studied in darkfield illumination the stained neutrophil granules appear like small black grains but

in close adjacency there are other luminous granules. Also in this case two different kinds of granular are visible, adjoining one another. This observation has eluded *Eränkö* as well.

The eosinophil cell in darkfield illumination (Zeiss' cardioid condenser) is of great interest. Also here the dark drops are optically void and seem to be black. Adjoining the black drops, the pale drops are observed as small luminous balls. The dark drop is often surrounded by a luminous ring, the small luminous ball forming the »stone« of the ring. In a cell, observed in transmitted light, the luminous ring corresponds in order of size to a dark drop and its surrounding hyaloplasma. In the living cell, in darkfield, the luminous ring is often jerked forward. In transmitted light the dark drop, with surrounding hyaloplasma, makes a similar movement together with the pale drop. If a living eosinophil cell is crushed the dark drop, the hyaloplasma ring, and a small pale ball, as the »stone« in the ring, may be distinguished in a minute part of the coagulated cytoplasma. Thus, identical structures, with two kinds of drops, may be seen in the eosinophil cell under greatly varying conditions. These observations, which support the conception of the double drop system are unknown to *Eränkö*.

Studying an eosinophil cell in transmitted light, the dark drops seem to become pale when the tube is raised. In several parts of the cell the transition takes place symmetrically, but an asymmetry is distinctly observed here and there in the same cell. Simultaneously with a black drop becoming white, as the tube is raised, a shift to the side occurs. I have shown this phenomenon, which implies that two drops are situated close to one another, to several experienced researchers, and our conceptions were found to be analogous — but *Eränkö* cannot see it.

Eränkö says that only dark granules are visible in the eosinophil cell in a phase contrast microscope. I can see distinctly both dark and pale drops in the phase contrast microscope (Zeiss or Wild) in the same way as in transmitted light, but the contrast between black and white is more marked. Why *Eränkö* has arrived at a different result is rather puzzling.

Some physical speculations form the ground upon which *Eränkö* bases his criticism regarding my conception of the double drop system in the protoplasma. He does not take into consideration that, in my endeavours to elucidate the optical phenomena which are caused by the granular substances of the cell, I have co-operated with *Lennart Simons*, Professor of Physics, and his three graduated assistants.

Eränkö's investigation is based on one single form of cell, the eosinophil granuleocyte, in transmitted light. The cell is coarse in structure and in many respects very suitable, notwithstanding the study being rendered somewhat more difficult due to the highly refractive hyaloplasma. There are many forms of cells, as may be de-

duced from what has been said above, which, in the darkfield microscope, are extremely valuable in support of the opinion that a double drop system exists in the protoplasma.

I am glad that this problem has come under discussion, as the understanding of the double drop system in the living cell takes a prominent place in elucidating the microscopical structure of the cell. *Eränkö's* paper shows, in my opinion, that these problems are probably too difficult for a novice.

Summary.

Eränkö's remarks regarding the existence of a double drop system in the protoplasma are refuted. A description is given of certain phenomena, indicating the presence of a double drop system in the cytoplasm of the living cell, which have not been observed by *Eränkö*.

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MYXOMA—MYXO-SARCOMA

A SURVEY AND A FOLLOW-UP EXAMINATION

By *Olav Anthun.*

(Received for publication April 29th, 1949.)

A myxoma is a tumour consisting of mucous tissue which, in its most characteristic form, makes up the structure of the umbilical cord. The tissues of this tumour consist of star-shaped cells with long offshoots and inter-cellular substance which is homogeneous and plentiful, giving a positive reaction to the stain mucicarmine. *Virchow* was the first to notice this tumour and to include it in his classification of tumours. In his opinion it was derived from mesenchymal maternal tissue because it was to be found in the body in places in which other tissues of mesenchymal origin existed, for example the fibrillary connective tissue and fatty tissue in fascias, the interstices of muscles, blood vessels and lymphatic glands. *Virchow* considered this tumour benign, for though it might recur after an operation, it did not form metastases. Later on he believed that it was benign from the outset and subsequently underwent secondary changes, becoming malignant. The notion of malignant degeneration was thus introduced into pathology, and the myxo-sarcoma was given its place in the classification of tumours.

There is little to be added to *Virchow's* description of these tumours, interest in which has not been very great. This is so, probably, because they are relatively rare, and it is therefore difficult for a single observer to collect several such cases. The publications I have found on this subject have for the most part been isolated reports, and I have discovered only one comprehensive, American study.

Meyenburg (1929) has pointed out that on a microscopic examination of tumours of the sarcoma group it is not rare to find myxomatous areas, but this does not necessarily mean that a tumour is a myxo-sarcoma. For this to be so there must not only be mucous intercellular substance present, but also typical mucous cells. He held that mucous areas without cells were the products of degeneration, such as could

be found in mesenchymal tumours known as lipoma myxomatodes, enchondroma myxomatodes. *Ewing* found myxomatous tissue in a series of epithelial tumours, and it was interpreted as a product of secretion. *Durante* (1902) and *Willis* (1948) have drawn attention to the similarity in many ways of oedematous areas (such as are found, for example, in fibromas) to mucous tissue.

The pathologist is often hard put to it in deciding whether a tumour is a myxoma or a myxo-sarcoma. In general terms it may be said that a myxoma is a well-circumscribed tumour, of uniform structure and often encapsuled. A myxo-sarcoma presents a quite different picture, with its numerous star-shaped cells of various sizes and with long offshoots, giving the inter-cellular substance a thready appearance. The chromatin content of the cells varies greatly, but it is difficult to find mitoses. The tumour is permeated by fibrillary connective tissue rich in capillaries and by fat which appears partly as isolated fat cells, partly as heaps of them. The tumour may be completely or partially enclosed in a capsule, and not infrequently there is macroscopic evidence of growth of the tumour by infiltration. On a microscopic examination all the different grades between a typical myxoma and a myxo-sarcoma can be seen. *Hertzler* (1931) maintained that whenever a lipoma was situated in fascia the tumour must be regarded as malignant because of the constant recurrences and because it became more and more rich in cells. He concluded that the transition from deep-seated lipomas and lipomyxomas on the one hand to lipo-myxo-sarcomas on the other hand was unnoticeable.

With regard to the mode of origin of a myxo-sarcoma, *Ewing* (1935) was unable to accept *Virchow's* conception of »malignant degeneration«. *Ewing* was in favour of the metaplasia theory. Fatty tissue and mucous tissue, according to this theory, arise by a process of metaplasia either from the adventitial cells of the blood vessels or from the endothelium of the capillaries. The formation of fatty tissue represents the first phase of the process of metaplasia, and the formation of mucous tissue follows. *Ewing* was emphatic in his opinion that a myxoma consists of living cells and has nothing to do with mucous degeneration which represents dead tissue. Besides, a myxo-sarcoma is always closely related to fascia sarcomas (lipo-sarcomas and fibro-sarcomas). *Boyd* (1943) was of the same opinion, maintaining that a myxo-sarcoma can hardly be regarded as representing an independent group of tumour. The appearance of myxomatous tissue in a mesenchymal tumour, whether it was or was not benign, means that the tumour has become malignant.

Heredity has not been found among the factors predisposing to the development of these tumours. I have been unable to find definite information concerning the weight to be attached to the influence of trauma, single or multiple, or to chronic irritation maintained for a considerable time. In *Ewing's* article, however, he emphasizes the

great importance of trauma, notably when these tumours are situated in the lower limbs.

The situation of these tumours in the body is most varied, the upper and lower limbs, the trunk, head and neck, and internal organs such as the kidneys, uterus, and ovaries (*Thibaudeau* 1935). *Thoma* (1944) found that the tumours situated in the upper and lower jaw presented a characteristic radiographie picture showing trabecular rarefying of the substance of the bone («honeycomb form»).

Treatment began, naturally enough, with exsision of the tumour, and such surgical treatment was sufficient when given early. But the great tendency to relapse was soon recognized. *Volkman* recommended early amputation if the operation was not radical. No one has recommended radiological treatment by itself, and we have gradually come to the present-day conclusion that the best treatment is a combination of surgical and radiological measures.

Among earlier publications, mention may be made of the 2 cases of myxo-sarcoma in muscles described by *Küttner* (1913). In 1916, *Robertson* collected 51 cases which had been published up to that date. In these cases the tumours were situated behind the peritoneum, in the kidneys and the pleurae, as well as in the muscles. *P. Bull* (1918) reported a case in which a gigantic, retro-peritoneal tumour, weighing 15.5 kg., was removed by operation. On account of its slow growth it was taken to be benign, but metastases developed later on in the lungs. A microscopic examination revealed a myxo-fibro-chondro-sarcoma. Several publications from French sources have dealt with these tumours in the period 1926—1931 (*Bérard*, *Creyssel*, *Desjardes*, *Fruchaud*, *Travenière*) and they were known as conjonctivomas. Such tumours are composed of several different kinds of tissue and are also called fibro-myxo-lipo-sarcomas. *Fruchaud* has recorded two cases in both of which the tumour was removed by operation. A relapse in one of these cases necessitated amputation. Of the total of 18 cases recorded by *Bérard* and *Peycelon*, 15 underwent surgical and radiological treatment and 8 survived. The 2 cases receiving only radiological treatment ended fatally. Radical, surgical removal of the tumour followed by radiological treatment was advocated by these two observers. *Hirschfeld* (1929) has described 4 cases of lipo-sarcoma containing myxomatous tissue. *Thibaudeau* (1935) has collected 50 cases among which were 9 cases of myxo-fibroma. All these 9 were alive after surgical treatment combined in some cases with radiological treatment. There were 3 cases of myxo-lipo-sarcoma and 39 of myxo-sarcoma. Among the 31 cases of sarcoma receiving surgical and radiological treatment there were 10 patients who survived, but the observation period was rather short in some of these cases. All the 4 patients receiving surgical treatment and treatment with radium died, and of the 3 patients receiving only radiological treatment, 2 died, and the sole survivor was not rid of the tumour. *Ewing* (1935) has recorded

2 cases, and in a review of these tumours he has indicated that they are more common in women than in men and in the age group 40—60 years than at other ages. He found the mortality highest when the lower limbs were involved, and he regarded as radio-sensitive those varieties in which there was much fatty tissue.

Usadel (1937) has paid special attention to the malignancy of the myxomas, and has come to the conclusion that the tumour recurs even when, on the histological evidence, it has seemed to be benign. The malignancy of the tumour grows with attempts to remove it radically by operation. Among his 23 cases were 7 of pure myxoma, 4 fibromyxomas, 8 myxo-sarcomas, 4 myxo-fibro-sarcomas, 1 myxomatous, fuso-cellular sarcoma and 1 myxo-osteoid sarcoma. Among his 25 cases there were only 3 which did not recur. The treatment given was surgical and radiological. Jönsson (1938) found 5 cases of myxolipo-sarcoma in muscles and fascia in the records of *Radiumhemmet* in the period 1920—1936. The treatment consisted in every case of excision through healthy tissue. Resurrections of the disease in 3 of these cases necessitated a new operation followed by radiological treatment. There was only one survivor among these patients. Both the patients receiving pre-operative radiological treatment were alive. Jönsson held that the efficacy of radiological treatment was in doubt because the microscopic examination of the tumour tissues showed no sign of damage from it. Said and McGinnes observed no effect from radiological treatment in 2 cases. Stewart (1937) found that the myxo-sarcomas were to some extent amenable to radiological treatment.

Own Investigations.

My material comes from the Ullevaal Hospital in the period 1929—1948, in the course of which I have found a total of 11 cases. Nine of the patients were in surgical wards and 2 in the Dental-Surgical Department. Three of the cases were observed before 1940, the remainder belonging to a later period. Five of the patients belonged to the age group 40—50 years. There were 2 patients under the age of 30. The youngest patient was a boy, aged 6 years, the oldest a man of 70. There were 8 women to 3 men. On perusing the anamnetic data I could find no record of familial predisposition or of trauma. The length of the interval during which the patients had noticed their tumours varied greatly. One woman stated that she had noticed the swelling for 13 years and others for only a year or two or for only a short time. In one case the tumour was discovered by accident when the patient, a woman, was in hospital receiving treatment for some other condition. Some patients stated that the tumour had grown somewhat »of late«, and others said that it had shown no change. The size of the tumour varied from that of a pea to that of a nut, a hen's

egg, an orange, or a loaf of bread. On macroscopic examination the tumour was soft, and its fluid contents were viscid. A microscopic examination was made in every case, and the microscopic preparations have again been examined. In one case this preparation could not be produced, so I had to confine myself to an earlier description of it.

On the microscopic examination I found in 5 cases benign-looking myxoma tissue, in 1 case slightly atypical cells and mucous tissue permeated by fibrillary connective tissue and fatty tissue indicating the diagnosis of lipo-myxo-fibroma. In 4 cases typical sarcoma tissue was found with mucous tissue, connective tissue and fatty tissue in varying proportions. Hence the diagnosis of fibro-lipo-myxo-sarcoma in 2 cases, lipo-myxo-sarcoma in 1 case, and myxo-fibro-sarcoma in 1 case.

The tumours were situated in the lower jaw, the upper jaw, the naso-pharynx, in the deep subcutaneous tissues of the left axilla, the vulva, the left inguinal region, the anterior aspects of the right and left thighs under their fascia, the popliteal space and posterior aspects of the left thigh, the sole of the right foot and the nape of the neck respectively.

In two cases a macroscopic probability diagnosis of myxoma tissue was made at the operation, and in two cases it seemed at the time of the operation that radical removal of the tumour was impossible. In one case the tumour was considered inoperable from the outset, and the operation was limited to a small exploratory excision.

On my follow-up examination I learnt that 3 of the 11 patients had died, and that a post-mortem examination had been made in one of the fatal cases. I have succeeded in ascertaining the present condition of all the 8 survivors. In 6 cases the successful removal of the tumour by operation was not followed by a recurrence during an observation period of 1—12 years. In 4 cases there was a recurrence, and a new operation was performed with the result that one of the patients showed no recurrence while the others did so again, and one of these patients was operated on without avail for the third time. In addition to surgical treatment, 4 patients received radiological treatment which in 3 of these cases was not given directly after the operation, but on recurrence of the tumour. The full tumour dose (see table) was given. Direct radiological treatment of the tumour itself was given in the inoperable case with the result that for a short time there was complete disappearance of the tumour which had recurred. But this did not prevent the development of metastases in the internal organs later on. One patient was alive a year and a half after the operation and showed no local recurrence, but there were general metastases. Among the 3 who died there was one who was cachectic without showing signs of local recurrence. Unfortunately permission for a post-mortem examination was refused. Another patient died at home, the cause of

Table.

Nr.	Sex	Age	Localization	Treatment		Result	Observation period	Biopsy
				Operative	Röntgen			
1	♂	44	Maxilla	Exeochleatio		No recurrence	1½ years	Myxoma
2	♀	45	Left axilla	Excision		No recurrence	3 years	Lipo-myxo-fibroma slightly atypical
3	♀	56	Vulva	Excision		No recurrence	12 years	Myxoma
4	♀	46	Right thigh	Excision		No recurrence	4½ years	Myxoma
5	♀	70	Left thigh	Excision		No recurrence	12 years	Myxoma
6	♀	50	Planta pedis	Excision		No recurrence	1 year	Fibro-lipo-myxo-sarcoma
7	♀	62	Right inguinal region	Operated on thrice	3600 r.	Death		Myxo-fibro-sarcoma
8	♂	70	Nape of the neck	Operated on twice	2 seances	Death		Fibro-lipo-myxo sarcoma
9	♀	42	Left popliteal space	Operated on thrice	Several seances 2700 r. & 3500 r.	Alive with pulmonary metastases	1½ years	Lipo-myxo sarcoma
10	♀	28	Mandibula	Operated on twice		No recurrence	1½ years	Myxoma
11	♂	6	Naso-pharynx		3450 r. + 3450 r. 2 fields	Death		Myxo-sarcoma

death being registered as heart disease. In neither of these cases can we be sure that there were no general metastases. The third death was due to metastases in the lungs and spine. The post-mortem examination showed no local recurrence.

The conclusion to be drawn from this follow-up investigation must therefore be that the radical removal of myxoma tumours should always be undertaken. If the microscopic examination shows benign

tumour tissue, nothing more need be done. If the pathologist reports that the tumour is a sarcoma, radiological treatment should be given at once even if it seems to have been radically removed at the operation. Radiological treatment should not be deferred till recurrence has occurred. It is also well to remember that when the tumour is situated in the interstices of the muscles of the lower limbs, radical removal of it is usually difficult or impossible, and the question of amputation of the limb should therefore be raised at once.

Summary.

The pathogenesis and localization of, and earlier publications on, myxoma and myxo-sarcoma tumours are reviewed by the author who gives an account of his own material consisting of 11 cases. The tumour was a pure myxoma in 5 of these cases, a lipo-fibroma in 1, a fibro-lipo-myxo-sarcoma in 2, a lipo-myxo-sarcoma in 1, a myxo-fibro-sarcoma in 1 and a myxo-sarcoma in 1 case. The myxomas were removed by operation. In a case of recurrence, a later operation was followed by freedom from recurrence. One of the cases of myxo-sarcoma was inoperable. The tumour disappeared after radiological treatment, but metastases developed early and the patient died. Among the 4 patients undergoing a radical operation there was one who remained free from recurrence. In the other cases there was a recurrence requiring a second operation which was followed up by radiological treatment. It was ineffective, for metastases proved fatal.

The author comes to the conclusion that radical removal is necessary for pure myxomas and lipo-fibro-myxomas. The myxo-sarcomas are relatively radiologically sensitive, but radical surgical treatment is necessary and should be followed by radiological treatment.

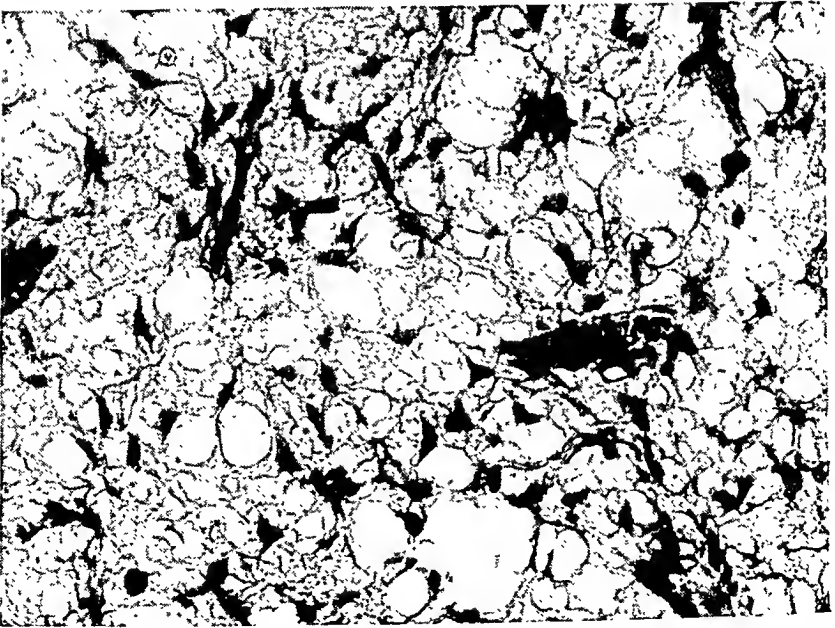
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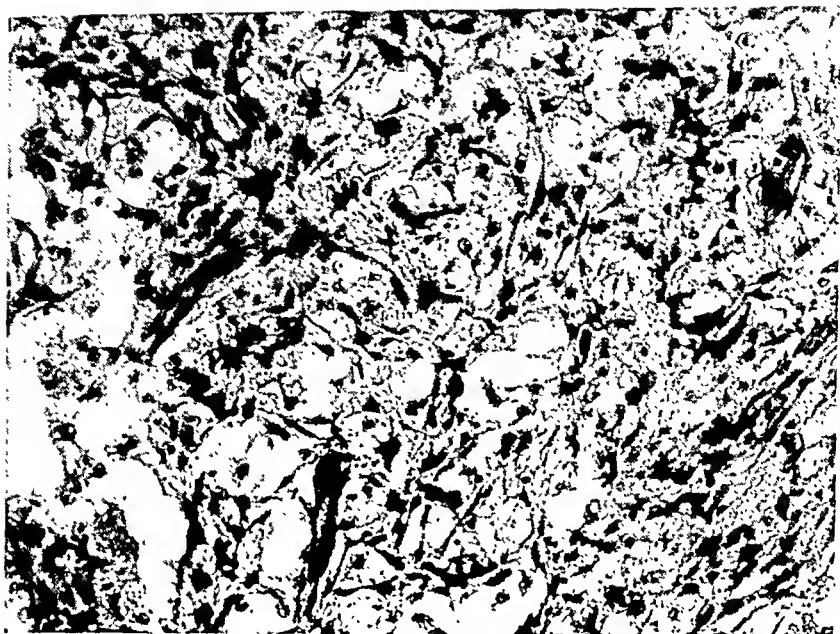
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Case 10.
Skiagram of lower jaw.



Case 8.
Fibro-lipo-myxo-sarcoma. $\times 350$.



Case 7.
Myxo-fibro-sarcoma. $\times 200$.



Case 9.
Lipo-myxo-sarcoma. $\times 200$.

THE SIGNIFICANCE OF HISTOLOGICAL GRADING IN THE PROGNOSIS OF CARCINOMAS IN THE TRUE ORAL CAVITY

By *Reidar Eker* and *Rolf Weyde*.

(Received for publication May 14th, 1949.)

In a previous paper discussing The Norwegian Radium Hospitals observations during the period 1932—1942, consisting of 386 cases of carcinomas of the true oral cavity, all histologically verified, one of us (Weyde) came to the conclusion that the primary tumor in most cases decides the fate of the patient. In other words, with the therapeutical methods employed, the decisive facts in the prognosis of carcinomas of the true oral cavity are identical to those determining the cure of these carcinomas by radium treatment.

The present paper is an attempt to find whether a more detailed histological classification, and particularly, a grading according to Broders' method, can give useful prognostic facts or information regarding the anticipated reaction to irradiation of squamous cell carcinomas of the true oral cavity.

The modern grading of squamous cell carcinomas was originated by Broders' in his works 1925 and 1927. In a number of works, particularly by Americans, the grading of carcinomas of the true oral cavity has been done according to this classification and it has been found useful in deciding the most suitable method of treatment in individual cases as well as for the prognosis. (H. E. Martin, J. J. Duffy and others).

Besides the ordinary squamous cell carcinomas there is, in the oral cavity, a number of other types of carcinomas of which a few are supposed to derive from the squamous epithelium. Ribert and Krompecher have described these as »basal cell carcinomas«. Others have described them as »undifferentiated carcinomas«. Ewing and other American pathologists like Quick and Cutler designate them transitional cell carcinomas.

The Norwegian Radium Hospital's Data.

These comprise all histologically verified cases of carcinoma of the oral cavity during the 10-year period 1932—1942 with an observation period of 5—15 years. The same material, ungraded, has been reviewed previously from clinical point of view, by one of us (Weyde) and part of it has been used by R. Bull Engelstad in a work dealing with the results of surgical treatment of cervical lymph node metastases.

All biopsies which were not from the primary tumor and those which had been treated by irradiation, or were too small, or technically unsuitable in other ways, have been eliminated in the preparation of the present work. There remain 210 biopsies. The patients are divided into the following groups: Free part of the tongue 56; Base of the tongue 41; sublingual region 35; bucca 33; gingiva 29; palate 16.

Method and definitions.

All sections are as a rule fixed in Formalin. Most of them are stained in haematoxylin-eosin, others according to Masson's trichrom-staining, and some by the van Gieson method or with mucicarmin or by silver impregnation after Laidlaw.

In the histological examination certain morphological facts were considered, such as tendency to infiltrate, amount and type of atypical nuclei, presence of cornification and stratifying cells as well as the size of the cells. The proportion of abnormally large nucleoli present was also investigated — these are referred to as »undifferentiated« cells. The histological examination has been carried out without clinical information.

Infiltration: The degree of infiltration was estimated and graded according to slight, average or strong tendency and has been classified in the tables as 1, 2 or 3.

The degree of cornification, the amount of *atypical nuclei* and *stratifying cells* were estimated in the same way and classified 1, 2 or 3.

We distinguished between small, medium, large and polymorphous *types of cells*.

Finally, we investigated the proportion of undifferentiated cells with pathologically enlarged nucleoli, including cells with mitosis. These were divided into groups 1, 2, 3, or 4 according to Broders' classification: group 1 includes 0—25 % undifferentiated cells, group 2: 25—50 %, group 3: 50—75 % and group 4: 75—100 %. From 200—500 cells from different areas of the section were counted, counting especially the proliferating strands of tumor cells.

Metastatic tendency. From clinical point of view, an important, probably the most important criterium in deciding the clinical malignancy of a tumor is its metastatic tendency. A tumor that

metastasizes to the regional lymph nodes before the primary lesion can be diagnosed, is considered to be the most malignant type. One that metastasizes late, or not at all, is the least malignant. An indication of the metastatic tendency of a tumor is found by dividing the material into the ordinary 3 stages according to the presence and extent of lymph node metastases on the admission to the hospital. This method is used in Table 2. Further information about the metastatic tendency is obtained by observing how many patients, who were admitted in Stage 1, without lymph node metastases, develop these, operable or inoperable, later. This will be seen from Table 3. Based upon these observations and the time when metastases occur and their rate of growth, we have grouped the tumors according to slight, moderate and strong metastatic tendency.

Primary effect of irradiation. We have used the expression primary effect of irradiation instead of radio-sensitivity, which may often have a confusing significance. It is used to describe the clinical reaction of a tumor, based upon following up and examination for 6 months after termination of the treatment. »Good primary effect« means that the patient became primary symptom free after external irradiation only. »Moderate primary effect« means that the tumor has shown moderately good response to external irradiation, but that there was a small residual tumor left which could be destroyed by interstitial radium treatment. In a few cases the residual tumor was first fulgurated and radium needles inserted into the wound bed in the same sitting. »Slight primary effect« applies to tumors that have shown little or no response to irradiation and where primary freedom from symptoms could not be obtained.

This classification has been used for small and large tumors respectively, and the comparison has been made on tumors of approximately the same size. The expression »radiocurable« has only been used for tumors which remain symptom free for 5 years or more.

We have distinguished between 1. Cornifying squamous cell carcinomas and 2. »Other types of carcinomas« of the true oral cavity. The basis for this classification has been given under the respective paragraphs.

Results.

1. *Cornifying squamous cell carcinomas.* This group comprises all the ordinary cornifying squamous cell carcinomas. It is a heterogeneous group of tumors of entirely dissimilar differentiation and includes also anaplastic tumors with only slight degree of cornification. (Phot. no. 1, 2, 3, 4).

Table 1 gives the results of comparison between a number of morphological and clinical factors, i. e. mortality, metastatic tendency and primary effect of irradiation. It is based upon the entire material regardless of the localisation of the tumor.

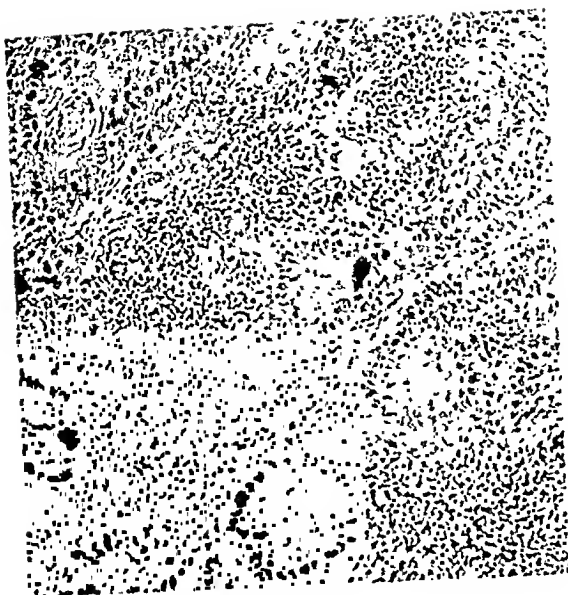


Fig. 1.
Squamous cell carcinomas. Grade I. $\times 140$.



Fig. 2.
Squamous cell carcinomas. Grade II. $\times 280$.

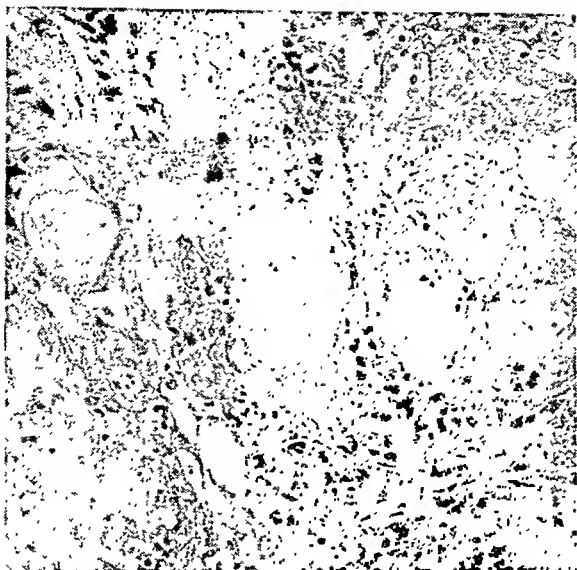


Fig. 3.
Squamous cell carcinomas. Grade III. $\times 140$.

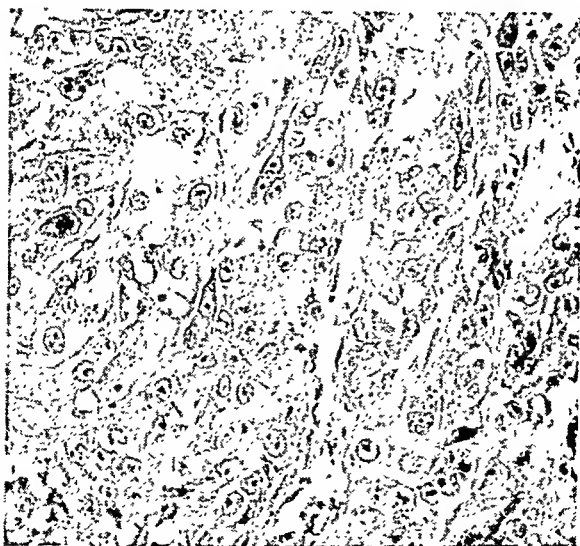


Fig. 4.
Squamous cell carcinomas. Grade IV. $\times 280$.

Table I.

Relation between morphological criteria and clinical factors: 5 years results,
metastatic tendency and primary effect of irradiation.

Morphological factors	No	Dead from cancer	5 years s. free	Metastatic tendency				No	Primary irradiation effect				No
				Small	Medium	Large	No		Small	Medium	Large		
Infiltration	1	66.6	33.4	100	0	0	9	0	33.3	66.7	6		
	2	47.1	52.9	67.5	3	27.5	10	41.2	15.7	43.1	51		
	3	12.5	87.5	37.3	13.7	49	110	31.9	21.1	41	83		
Atypia	1	61.5	38.5	80.4	13.3	6.3	15	23.1	30.7	46.2	13		
	2	33.3	66.7	67.3	3.8	28.9	52	39.5	20.1	48.1	51		
	3	11.9	88.1	32.6	11.1	57.3	92	18.6	18.3	33.1	82		
Cornification	1	19.2	80.8	50.3	12.1	57.6	33	50	18.8	31.2	32		
	2	25.7	74.3	12.9	1.7	52.1	12	10.5	11.1	15.1	12		
	3	22.5	77.5	58.3	13.1	28.6	81	36.0	25.3	38.7	75		
Intercellular bridges	1	20.0	80	37.2	11.9	50.9	51	15.2	19.1	35.7	42		
	2	21.6	78.4	31.1	19.5	46.1	41	26.3	23.8	49.9	42		
	3	25	75	65.6	1.5	29.9	67	16.1	18.5	35.1	65		
Cell-type { Small Medium Large Polymorphous		16.7	83.3	71.1	0	28.6	7	50	20	30	10		
		28.1	71.9	63.1	8.5	28.1	82	26.9	26.9	47.2	83		
		17.3	82.7	12.9	11.2	12.9	35	11.7	16.6	11.7	21		
		7.7	92.3	20	20.0	60	25	59.1	9.1	31.8	22		

Without giving details, the table shows that the tendency of infiltration as well as the degree of atypia have a remarkable and systematic correlation to mortality, metastatic tendency and primary effect of irradiation. As for the effect of irradiation the most obvious connection is between the »infiltration tendency« and the amount of undifferentiated cells. In the group including slight infiltrative tendency 0 % shows slight primary effect of irradiation, while 66.7 % show good primary effect. In the group including strong infiltrative tendency 34.9 % show slight and 41 % show good primary effect of irradiation. For atypical nuclei the proportions are similar but more difficult to determine.

It is worth noting that the estimated number of areas in the sections showing cornification is of comparatively small importance in estimating the malignancy of the tumor or its reaction to irradiation. The same applies to the stratifying epithelium in the sections. It may be pointed out that only when extreme polymorphous cells are present some relation to the mortality and the metastatic tendency may be demonstrated. As for the size of the cells and the primary effect of irradiation, it is noticeable that any large proportion of irregular cells, whether small, large or polymorphous, will increase the percentage of slight primary effect of irradiation and consequently decrease the percentage of good primary effect, but the result is rather unconvincing.

We have also estimated the frequency of mitosis in the squamous cell carcinomas with cornification. The amount of mitosis in 10 areas chosen at random was counted and their density was estimated by counting the number of cells in 3 to 5 areas. The results were calculated at the rate per thousand. The value of this calculation is of course only of relative importance but might give sufficient indication of the frequency of mitosis. In relation to the metastatic tendency it was found that in carcinoma of the tongue with slight, moderate and strong metastatic tendency the index of mitosis was, respectively, 3.09 (0.33—16), 2.67 (0—11) and 2.31 (0—12). The figures in brackets indicate the dispersion margin. For carcinomas with slight, moderate and good primary effect of irradiation the index of mitosis was 5.06 (0—31), 4.95 (0.8—12) and 3.42 (0—16).

Taking into consideration the wide dispersion margin, any marked relation between the index of mitosis and the metastatic tendency cannot be proved. With an increased primary effect of irradiation the frequency of mitosis is found to be diminishing. This is in accordance with the relation between the histological grade and the frequency of mitosis — to which reference will be made later. But with this slight difference and the wide dispersion margin it appears that the indices for mitosis alone are of minor importance in classifying the biopsy.

We have classified the cornifying squamous cell carcinomas ac-

cording to Broders' method, based on the number of »undifferentiated« cells (see page 2) but are inclined to consider the infiltrative tendency to be of great importance. All borderline cases, that is all cases with nearly 25 %, 50 % or 75 % »undifferentiated« cells, were automatically placed in the higher grade when a strong infiltrative tendency was apparent. For example: cases with nearly 50 % »undifferentiated« cells were placed in grade II where the extent of infiltration was 1 or 2 and in grade III where the extent of infiltration reached 3. Other facts were not considered in the classification.

Table 2 shows a close relation between the grade of the tumor and the clinical stage of the patient at the time of admission. In grade I all patients are in stage I, in grade II about 80 %, in grade III — 49.1 % and grade IV only 20 %. These figures are taken from the entire material, but, as will be seen from the table, the same proportions apply when the material is divided into separate groups for carcinoma of the tongue and for buccal, gingiva and palate.

Table 3 shows the frequency of regional lymph node metastases which appear subsequent to the patients' admission in stage I. It will be seen that grade I patients do not develop metastases, while 70 % of the patients in grade IV do so subsequently.

Table 4 shows evidence of the tumors' metastatic tendencies and their relation to the histological grade. There is a marked difference in the metastatic tendency of the various localizations as well as for the material as a whole. The metastatic tendency is only slight in grade I and II while it is strong in grade III and IV.

A comparison between the grade and the primary effect of irradiation gives an interesting result. Table 5 gives a clear indication that in tumors of the higher grades, that is the most anaplastic tumors, the chances of obtaining a good primary effect of irradiation are diminished considerably. Calculated on the entire material, 9.5 % showed slight and 66.6 % showed good primary effect of irradiation in grade I, while in grade IV the corresponding figures were 70 % and 13.4 %. The figures for grade II and III fall in between. If the small and the large tumors are examined separately (the latter with a diameter of 5 cms. or more) the figures are somewhat smaller but the same tendency is apparent. As may be expected, the primary effect of irradiation on the smaller tumors shows a distinct relation to the grade, but it is surprising to find it to such a large extent in the larger tumors.

The same relationship was apparent also in the previously mentioned comparison of the individual morphological factors and the primary effect of irradiation (table I).

In this classification the localization of the tumor has not been taken into consideration, as any essential difference in the relation between the localization and the primary effect of irradiation could not be proved. In *gingival cancer* certain special conditions have to be considered as some of the tumors may have caused destruction of the

Table 2.

Relation between histological grade and clinical stage on admission to the hospital.

Grade	No	% St I	% St II	% St III	No	% St I	% St II	% St III	No	% St I	% St II	% St III
I	15	100	0	0	10	100	0	0	25	100	0	0
II	32	87,5	6,3	6,3	17	76,5	17,6	5,9	49	83,7	10,2	6,1
III	37	43,2	27	29,8	22	60	13,6	26,4	59	49,1	22,8	28,1
IV	23	26,1	26,1	47,8	14	7,1	64,3	28,6	37	19,0	40,5	40,5
Tongue				Bucca Gingiva Palate					Total			

Table 3.

Percentage of patients admitted in stage I, who later developed regional lymph node metastases, grade I—IV respectively.

Grade	Tongue		Bucca Gingiva Palate		Total	
	No St I	% Later met	No St I	% Later met	No St I	% later met
I	14	0	8	0	22	0
II	26	34,6	13	33,3	39	33,3
III	14	50	13	23	27	37
IV	5	80	1	?	6	71,7

Table 4.

Relation between histological grade and metastatic tendency.

Grade	No	Tongue			No	Bucca Gingiva Palate			No	Total		
		Small	Medium	Large		Small	Medium	Large		Small	Medium	Large
I	14	% 100	% 0	% 0	10	100	0	0	24	100	0	0
II	30	77,7	13,3	10	17	76,4	5,9	17,7	47	76,5	10,5	13
III	31	16,1	19,4	64,5	22	45	13,6	41,4	53	28,3	17	54,7
IV	21	4,8	14,3	80,9	14	7,1	0	92,4	35	5,7	8,6	85,7

Table 5.

Primary effect of irradiation on the tumor in relation to its histological grade, small and large tumors respectively.

Grade	% Small irradiation effect			% Medium irradiation effect			% Great irradiation effect			Total
	Small	Large	Total	Small	Large	Total	Small	Large	Total	
I	5,5	33,3	9,6	27,8	0	23,8	66,7	66,7	66,6	21
II	7,4	42,2	21,8	25,9	21,1	23,9	66,7	36,7	54,3	46
III	31,6	63,6	51,9	21,0	15,2	17,3	47,4	21,2	30,8	52
IV	83,3	66,7	70	0	20,8	16,6	16,7	12,5	13,4	30

bone before the patient was first admitted. This, of course, decreases the effect of irradiation. The material relating to gingival cancer covers only 21 cases. In 12 of these the bone was affected. 9 cases showed slight effect of irradiation irrespective of the histological grade. As these cases make such a small difference in calculating the figures for the entire material in Table 5, they have all been included. We shall come back to the importance of bone destruction later on in connection with the 5 year result.

Table 5 also shows a remarkable relation between the size of the tumor at the time of admission and the histological grade. Highly differentiated tumors are, as a rule, small on admission while slightly differentiated tumors are large. This is even more noticeable when the tumors are divided into groups according to size: small, with a diameter of less than 2 cms., medium with a diameter of 2—5 cms. and large with a diameter of more than 5 cms. Of the small tumors 82.6 % are grade I and II and there is none in grade IV. Of the medium size 73.5 % are grade II and III. 16 % are grade I and about 10 % are grade IV. Of the large tumors 64.3 % are grade III and IV while only 3.1 % are grade I.

Here it would be of great interest to have detailed information about duration of symptoms previous to admission. But we feel that the patients' own observations are so unreliable that they are of little or no use as guidance. For this reason we have not found any outstanding difference in the duration of the disease in relation to the grade of the tumor.

There is, however, a certain relation between the *grade of the tumor and its macroscopical appearance*. As will be seen from table 6, the majority of all papillomatous tumors have a low degree of malignancy, the ulceroinfiltrative types have a high degree of malignancy and the papillomatous infiltrative types fall in between.

Table 6.

Histological grade — macroscopical appearance of the tumor.

Grade	No	% Papillomatous	% Papillomatous inf.	% Ulcero inf.
I	26	60	17,8	11,9
II	50	20	26,7	31,1
III	57	20	37,7	32,4
IV	37	0	17,8	24,2

Tumors developed from leukoplacia show no definite relation to the histological grade.

There also seems to be a certain relation between *the patients' age and the histological grade*, but our material is too small to draw definite conclusions. It is, however, remarkable, when the number of tumors of the histological grades I and II are compared with grades III and IV — it is found that of the age group under 60, 16 cases are grade I and II and 30 cases are grade III and IV. For the age group over 60 the corresponding figures are 60 and 22.

Table 7 shows the relation between the histological grade and the mortality. The calculation is based on our material taken as a whole and also on the individual groups according to the localization of the tumor. Based on the entire material the figures for 5 year cures in grade I to IV are 68.2 %, 30.9 %, 9.09 % and 0 % respectively with corresponding figures for mortality. Death caused by a tumor of the lower grades may occur at any time within the 5 year period, while it usually takes place during the first or second year if caused by a tumor of the higher grade.

When the rate of mortality from cancer of the tongue is compared with that of other localizations it will be found that tongue carcinomas of the lower grades have a higher percentage of cure. In grade I for instance, the mortality for tongue carcinomas is 10 % and for other localizations 50 %. This is due to the unfavourable results from irradiation in gingival tumors with bone destruction. If gingival tumors with bone destruction are excluded from the calculation, the figures for the group bucea, gingiva and palate, grades I—IV, are 50 %, 66 %, 12.5 % and 0 % respectively, for 5 years symptom free. If we add up the figures for grade I and II and then add those for grade III and IV separately, the resemblance to the figures for tongue carcinomas is even more noticeable. Grade I and II tumors of the tongue have 47.4 % 5 years symptom free and bucea, gingiva and palate 47.6 %. For grade III and IV tumors the figures for the tongue are 3.6 % and for the other localizations 7.7 %.

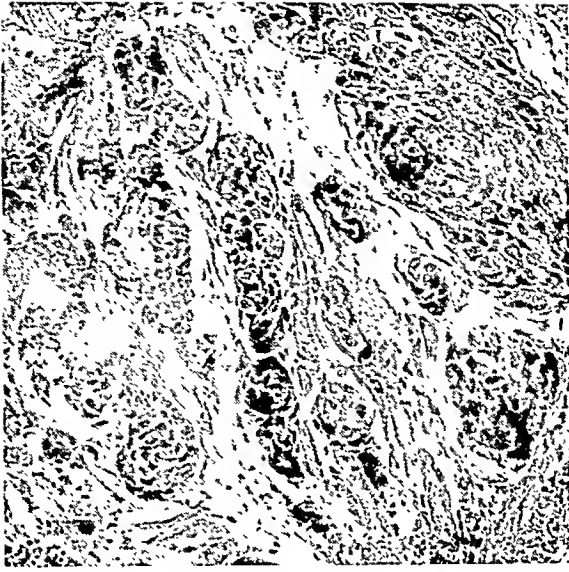


Fig. 5.

Anaplastic-parakeratotic carcinomas (AP). $\times 140$.

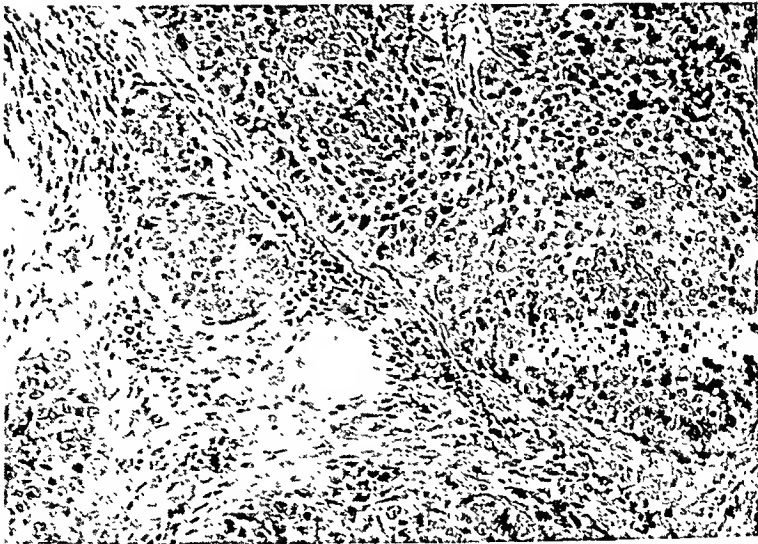


Fig. 6.

Anaplastic-spinous carcinomas (ASp). $\times 140$.

2. *Other types of carcinomas.* In 40 of the cases examined the squamous cell carcinomas were found to be morphologically dissimilar to the regular types of squamous cell carcinomas because of the domination of slight or medium differentiated cells. Broadly outlined.



Fig. 7.

Anaplastic-squamous carcinomas (ASq). $\times 140$.

it may be said that while the squamous cell carcinomas are similar to the highly differentiated cornifying layers in the multiple layers of the squamous epithelium, these tumors are partly similar to the least differentiated basal cells and partly to the cells in the medium layer.

These tumors might be described as transitional cell carcinomas.

In agreement with Glücksman and Spear (1945) we designate tumors consisting of undifferentiated cells as Anaplastic-parakeratotic tumors (AP). Tumors consisting of undifferentiated cells but with a certain number of cornifying centers we consider as Anaplastic-spinous when the area is dominated by cells of the middle layer, often with small cornifying centers (ASp). See phot. 5, 6, 7.

1. *AP tumors.*

This group includes 14 patients. The types of cells resemble those of the basal layer. As a rule they are slim, the cytoplasm is slightly basophile and the nuclei are oval with minute chromatine. Most of them are of the highly infiltrative types and grow in narrow bands. In all these types of tumors pathologically and large nucleoli are rare, and grading on the same principles as for the squamous cell carcinomas proved to be of no value for these groups.

Table 8 shows that none of these patients lived for more than 5 years, 2 died symptomfree within this period. The others all died within 3 years. Prognostically, this group closely corresponds to grade IV of the squamous cell carcinomas.

The table also shows that these tumors have a strong metastatic

Table 8.

Tumors in groups AP, ASq and ASp — Mortality, Metastasis tendency, Primary irradiation effect.

		AP.—14 pt.	ASq. and ASp. 26 pt.
<i>Mortality.</i>	5 years symptomfree	0 %	29 %
	Indeterminate	2 pt.	6 pt.
	Dead	100 %	71 %
<i>Metastatic tendency.</i>	Small	29 %	59,5 %
	Medium	14 %	4 %
	Large	57 %	36,4 %
<i>Primary irradiation effect.</i>	Small	38 %	22 %
	Medium	8 %	13 %
	Large	54 %	65 %

AP: anaplastic-parakeratotic carcinomas.

ASq: anaplastic-squamous carcinomas.

ASp: anaplastic-spinous carcinomas.

tendency. On admission, 43 % were in stage I, 21 % in stage II, and 36 % in stage III. Regarding primary result of irradiation, this group is in a special class, 54 % show good primary effect while 38 % show only slight primary effect. These figures do not illustrate, however, the extremely good effect of irradiation of some of the tumors.

Three tumors were found to be typical transitional cell carcinomas as described by Quick and Cutler: they were highly sensitive to irradiation. This type of transitional cell carcinoma can be identified with some certainty by its morphology. Otherwise we did not find any complex of morphological criteria giving definite indications as to the degree of reaction to irradiation to be expected in the individual tumors. Regarding the frequency of mitoses, calculated by counting 1000 cells chosen at random, we made the following observations: The five tumors with slight primary effect of irradiation had an average of 11.8 per thousand with a dispersion margin of 5—34. Those with good primary effect had 16.5 per thousand, also with a dispersion margin of 5—34. The latter had, on the average, a higher frequency of mitoses, but even these limited observations showed that the individual dispersion margin was so wide that the index for mitoses in this respect was of no avail for estimating the individual primary effect of irradiation.

2. ASq. and ASp. group.

This group includes 26 cases. 6 patients died from intercurrent disease, free from cancer symptoms. As will be seen from table 8, 29 % were symptomfree after 5 years, and 71 % died from cancer. Prognostically this group thus resembles grade II of squamous cell carcinomas.

On admission 65.1 % were in stage 1, 11.5 % in stage 2, and 23.6 % in stage 3. As will be seen from table 8 compared with table 4 and 5, the metastatic tendency for this group is higher than for grade II squamous cell carcinomas. The frequency of good primary irradiation effect is, however, greater than in squamous cell carcinomas of this grade.

The types of tumors referred to are more or less equally distributed in the various localizations of the true oral cavity.

In all three groups the ulceroinfiltrative types are dominating, only a few are papillomatous infiltrative and none is purely papillomatous.

Conclusions.

210 biopsies from non-irradiated tumors in the oral cavity were classified and graded according to a modification of Broders method.

1. We distinguished between the squamous cell carcinomas (170 cases) and other types of squamous cell carcinomas (40 cases). The latter types were divided into groups of anaplastic-keratotic tumors (AP), anaplastic-squamous tumors (ASq) and anaplastic-spinous tumors (ASp). While the cornifying squamous carcinomas could be graded on the basis of the percentage of »undifferentiated« cells without consideration of the infiltrative tendency, this was not practical for the other types of carcinomas.

2. For the cornifying squamous cell carcinomas it was possible to prove the existence of a relation between the histological grade and the following clinical observations:

- a. The size of the tumor on admission. Generally, tumors of a low grade were small and those of a high grade large (table 5).
- b. The macroscopical type of the primary tumor. Most of the papillomatous types were of low histological grade, the ulceroinfiltrative types were of the highest histological grade, while the papillomatous infiltrative types fell in between (table 6).
- c. Greatly increased metastatic tendency in progressively higher grades (tables 2, 3, 4).
- d. Primary effect of irradiation decreasing considerably in progressively higher grades (table 5).
- e. Radiocurability, that is 5 years freedom from symptoms, greatly decreasing with progressively higher grades (table 7).
- f. Prognosis of the lower grade tumors localized in the tongue is better than of bucca, gingiva and palate (table 7). This is probably due to the poor prognosis of gingival cancer caused by frequency of bone destruction.
- g. Probably there is a greater frequency of higher grade tumors in the lower age groups.

3. Other types of squamous cell carcinomas: The AP-group of tumors shows 100 % mortality although some of the tumors react

favourably to irradiation and their prognosis resembles that of grade IV of cornifying squamous cell carcinomas. The mortality of the ASp. and ASq. group tumors practically equals that of cornifying squamous cell carcinomas grade II, but the group shows a stronger metastatic tendency and also a more favourable reaction to irradiation.

Final remarks.

The prognosis of carcinomas of the true oral cavity radiologically treated is governed by a number of general and local factors, for instance the age and general health of the patient, the macroscopical type of the tumor, its size and localization as well as its histology (Ewing, Desjardens and others).

In this work we have examined the possibility of a tumor being classified according to an individual picture after Broders' method, to give some indication as to the expected effect of irradiation. The classification used indicates, without doubt, groups which react differently to irradiation. This applies to the primary effect, meaning the condition of the tumor after an observation period of 6 months, and the radiocurability, which may be defined as 5 years freedom from symptoms. One of the most interesting results of this classification was the fact that *with increased anaplasia in a cornifying squamous cell carcinoma it would be less likely that a lasting good effect of irradiation would be obtained*. The question seems to have attracted small attention till now, as it is too often taken for granted that anaplastic tumors also are sensitive to irradiation. Ewing (1929) states that »carcinomas are resistant in inverse proportion to the degree of anaplasia« and this opinion is still generally accepted although some pathologists, who have given the question special attention, have reached contradictory conclusions. Phillips (1930) writes in his excellent article on the grading of carcinomas of the oral cavity: »No direct relationship was found between the histology of buccal carcinoma and its response to irradiation«, Whitcomb (1943) writes: »it is doubtful if critical analysis of the results of radiation treatment of metastatic nodes or of the primary cancer will support the theory that radiosensitivity increases as the grade of squamous cell carcinoma,« and that »failure to differentiate a grade 4 squamous cell carcinoma from a sarcoma or other radiosensitive tumor metastasis can explain some unexpectedly good response to irradiation.«

Glücksman and Spear (1945) having investigated the question from a different angle, have come to the same conclusions as we have. Their method is to observe the reaction to irradiation in a series of sections of the tumor tissue and they have developed the most promising technique up to date of determining the sensitivity to irradiation of the tumor tissue. They emphasize very convincingly that the rapidity with which a tumor disappears clinically while under-

going treatment has little to do with the actual effect of irradiation. A complex of factors has to be considered, for instance the length of life of a cell, the speed of its mitotic division and its capacity of differentiation, a tumor which disappears completely, even if slowly, after irradiation, must in reality be considered the most sensitive to irradiation. This question is of fundamental importance and it is to be hoped that the term radio-sensitivity can, in the future, be given a more exact meaning.

There is no doubt that grading according to Broders' method is of importance in estimating the radio-sensitivity and prognosis in groups of tumors. As the radio-sensitivity of the tumor cells is influenced by multiple factors, it is surprising to find that grading based on a single biopsy can produce such distinct groups as in this instance. It apparently shows that grading according to Broders' method is very important in the statistical examination of material. In examining the individual tumor, however, we have been no more successful than anybody else in finding a complex of morphological facts which, in an individual biopsy, can give greater possibilities for the classification. There has actually been some doubt as to whether such criteria actually exist (Whitecomb, Richards) and it has been stated »that only a trial of irradiation will actually prove a given tumor in a given patient radiosensitive«. (Liljenkrantz). It is therefore of the greatest interest that Glücksman and Spear in making a series of biopsies during the radiological treatment of accessible cancers in only a few weeks, are able to indicate the prognosis in an individual case on the basis of few biopsies.

As already mentioned our results show that in cases of carcinoma of the oral cavity, irradiation therapy gives the best lasting results in the groups of highly differentiated squamous cell carcinoma. As far as the treatment of the regional lymph node metastases is concerned, grading may also be of some importance for the choice of mode of treatment. Tumors of the higher grades have a strong metastatic tendency, and in cases with manifest metastases they are inclined to recidivate in the operative area after total neck dissection. It would therefore appear natural to consider the advisability of performing prophylactic neck dissection in these highly anaplastic squamous cell carcinomas. This is true also because our results indicate that there is no logical reason to expect lasting cure following irradiation treatment in manifest metastases of these tumors.

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ON THE HEREDITY OF BLOOD FACTOR P.

By K. Henningsen.

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Following the discovery of blood factor P in 1927 *Landsteiner* and *Levine* established by family investigations, that the property was hereditary. 103 families with 498 children were examined the reactions being divided into classes according to receptor strength, and the results suggested inheritance determined by several allelic genes. It appeared that the property was not found in the blood of a child, unless it was present in the blood of at least one of the parents. The exact mechanism of inheritance, however, was not established. (11, 12).

Dahr and collaborators have investigated the heredity of factor P by twin and family investigations. Their material comprises 383 pairs of twins and 563 families with 2070 children. The investigations provide evidence, that the property is inherited as a simple Mendelian dominant, the presence or absence of the property being determined by two allelomorphic genes P and p of which the former dominates over the latter. (3, 4, 5, 6, 7).

Jungmichel has found conformity to this theory by investigation of 40 families (10), and further *Wolff* and *Jonsson* have published 501 mother-child examinations supporting this theory of inheritance. (17, 9).

Recently *Sanger*, *Lawler* and *Race* have published 85 family investigations, the results agreeing well with the theory. (13 a).

As to the inheritance of the receptor strength no thorough investigations have been published. The investigations of *Landsteiner* and *Levine* were performed with rather weak sera, and the classification with regard to receptor strength was attained by estimating the strength of the agglutination. *Dahr* has performed a number of titrations of the receptor strength in the family material, but the results of these investigations have not been published. Neither has *Jonsson*, who classify according to the varying absorbing power of the bloods (personal communication), published his results, and till now no de-

finite conclusions concerning the heredity of the receptor strength have been proposed.

The present paper comprises the authors investigations on the heredity of factor P per se as well as of the receptor strength based on mother-child and family investigations. Further the forensic aspect of blood factor P is presented as applied to cases of disputed parentage.

Technic and material.

The technic employed in this investigation has been described in detail in a previous paper. (8). A little less than half the mother-child determinations (330) and between one third and one fourth of the family material (364 determinations out of a total of 1228) have been subject to agglutination tests with animal anti-P serum and in a majority of cases with anti-P sera of human origin too. The remainder of the determinations has been performed by agglutination tests with an anti-P serum of animal origin, by titration against a human anti-P serum as well as by absorption with another human anti-P serum.

The material comprises 700 mother-child examinations originating from cases of disputed paternity, and further a family material totalling 1228 persons. This material originates from 54 families and comprises 281 matings with 727 children. In every family at least 3 generations have been examined, and every person has been tested according to the ABO, A₁A₂, MN and P systems. Further a great proportion of the material has been investigated with regard to the secretor system, Rh- and Lewis systems. The complete pedigrees of the families will be published later. The families have been selected preferably among families known to the author or his colleagues in order to reduce the likelihood of encountering illegitimacy. The last part of the material, however, comprising 123 matings has been selected a little less carefully, and among those matings 3 instances of illegitimate offspring have been found against none among the first 158 matings.

Results.

The results of the mother-child examinations are given in table 1.

Table 1.

Mother-child combination	Observed number	Calculated number	Observed percentage	Calculated percentage
P+ — P+	447	473	64	67.5
P+ — P—	103	80	14.7	11.5
P— — P+	82	79	11.6	11.3
P— — P—	68	68	9.7	9.7
Total	700	700	100.0	100.0

The observed and the expected distribution of 700 mother-child examinations.

Taking into consideration that the P receptor is not fully developed at birth the observed figures agree very well with a mechanism of inheritance conditioned by two allelic genes P' and p' , the former dominating over the latter. As the age of a great many of those children was less than 3 months, the problem of the inheritance of the receptor strength has been disregarded.

The distribution of the entire family material among the various blood group systems is tabulated in table 2.

Table 2.

Group or type	Total		P+		P—		Stand. error of percentage of P—
	Number	Percentage	Number	Percentage	Number	Percentage	
A ₁	425	34.7	346	81.4	79	18.6	±1.89
A ₂	111	9.05	88	79.3	23	20.7	±3.84
O	505	41.1	399	79.0	106	21.0	±1.81
B	137	11.15	107	78.0	30	22.0	±3.54
AB	50	4.1	39	78.0	11	22.0	±5.85
M	302	24.6	238	78.8	64	21.2	±2.35
N	287	23.4	242	83.7	45	16.3	±2.18
MN	639	52.0	499	78.0	140	22.0	±1.64
D	999	83.5	787	78.8	212	21.2	±1.29
d	199	16.5	164	82.4	35	17.6	±2.69
Total	1228	100	970	79.85	249	20.15	±1.15

The distribution within the family material among the various blood groups and blood types.

Although the material consists of comparatively few families, the observed frequencies of the different blood groups and types agree approximately with the average frequencies in the Danish population. Further no indication is found of any interdependence between factor P and the properties tabulated.

If two genes P' and p' are assumed with dominance of P' over p' , the phenotype P + comprises genotypes $P'P'$ and $P'p'$, and phenotype P — is $p'p'$. Thus the frequencies of P' and p' may be calculated knowing the frequency of genotype $p'p'$:

$$p' = \sqrt{P-} = \sqrt{0.2015} = 0.447.$$

$$P' + p' = 1, P' = 0.553.$$

If the distribution of the genotypes in the population is

$$P'^2 + 2P'p' + p'^2 \text{ then the offspring comprises}$$

$(P'^2 + 2P'p' + p'^2)^2$. From this the proportion of phenotypes in offspring from different combinations of matings may be calculated using the frequencies of P' and p' . In table 3 the observed and the calculated data are given together with the formulas for the calculations.

Table 3.

Matings	P +		Offspring	
	Formulas		P—	
P+ — P+	$P'^2(3-2P')$: 57.8 per cent.		$P'^2p'^2$: 6.1 per cent.	
P+ — P—	$2P'p'^2$: 22.1 » »		$2P'p'^3$: 9.9 » »	
P— — P—	0 : 0 » »		p'^4 : 4 » »	

Matings	P +		Offspring	
			P—	
P+ — P+: 180	430 (420)		49 (45)	
P+ — P—: 86	150 (161)		62 (72)	
P— — P—: 15	1 (0)		35 (29)	
Total : 281	581 (581)		146 (146)	

The calculated and observed distribution of matings and offspring with regard to the P factor. In parenthesis the expected number of children.

It is seen, that the figures agree very well with the assumed theory of inheritance.

One exception, however, is encountered, a mating P — — P — having a P + child. The complete blood group and type formulas of the family are the following:

Husband: A₂ N c d d P —

Mother: O MN CCD P —

Daughter: A₂ MN CCD P +

Son: A₂ MN C c D P —

It is seen, that the daughter besides being P + is homozygous C, while the husband of the mother is homozygous c, and further it was revealed, that the marriage of the parents took place about a month before the birth of the daughter. In view of these circumstances the author feels justified in excluding the family from the material and in considering it, not as an exception to the rule, but as an example of exclusion of paternity.

The genotypes of the various parental combinations and their offspring are shown in table 4.

Table 4.

Parents		Offspring
Phaenotype	Genotype	
P+ — P+	P'P' — P'P'	P'P'
	P'P' — P'p'	$\frac{1}{2}$ P'P', $\frac{1}{2}$ P'p'
	P'p' — P'p'	$\frac{1}{4}$ P'P', $\frac{1}{2}$ P'p', $\frac{1}{4}$ p'p'
P+ — P—	P'P' — p'p'	P'p'
	P'p' — p'p'	$\frac{1}{2}$ P'p', $\frac{1}{2}$ p'p'
P— — P—	p'p' — p'p'	p'p'

The genotypical combinations of matings and offspring.

As seen from the table the mating P + — P + produces both positive and negative children in the proportion 3 : 1, if both parents are heterozygous. As a negative child from a P + — P + mating is

an indication of the heterozygosity of the parents, those matings are selected having $P -$ children:

Matings	Children	
$P'p' - P'p'$	$P +$	$P -$
37	71	49
		Total
		120

As the matings are selected by the occurrence of $P -$ children, some heterozygous matings without negative children have evaded the enumeration. This error may be corrected using the following method by *Bernstein* cited by *Schiff* (14): the average number of children in each mating is $120/37 = 3.24$. The probability of a mating $P'p' - P'p'$ without negative children is $(3/4)^{3.24} = 0.394$. The probability of the observed matings is $1 - 0.394 = 0.606$. To get the real number of children originating from heterozygous matings the number of children observed has to be multiplied with $1/0.606$ viz. $120 \times 1/0.606 = 197$. One fourth of these children, 49.25, is going to be $P -$. Thus the observed number of 49 negative children agrees very well with the number calculated.

Yet another combination of matings, $P'p' - p'p'$, yields a possibility of testing the theory. The matings $P + - P -$ having negative children must be of this genotype. Yet some of these matings have only positive children in spite of the heterozygosity of the positive parent. In order to correct this source of error the matings with negative children only are excluded from the enumeration, as the probability is, that the number of these matings equals the number of matings of this genotype having no negative children. The remaining matings and their offspring are:

Matings	Children	
$P'p' - p'p'$	$P +$	$P -$
26	40	42
		Total
		82

As expected the proportion between positive and negative children is approximately 1 : 1.

Thus the present material gives further proof of the law of heredity of blood factor P stated by *Dahr*, that the inheritance is governed by two allelic genes P' and p' , and that P' dominates over p' . *Consequently a child cannot be $P +$ without having at least one $P +$ parent.*

As no agglutinin against $P -$ have yet been encountered, the term dominance may not be fully justified, but for practical purposes the theory is adequate.

Till now no regard has been paid to the variation of the receptor strength, which was determined by titration in a majority of the bloods from the family material. The titrations were carried out in dwarf test tubes by testing the corpuscles in question against progressive

dilutions of a human anti-P serum, $1/2$, $1/4$, $1/8$ and so on. As a control of the serum a positive blood of known receptor strength, preferably always the same, was included in every set up, and if necessary the results of the titrations were corrected as indicated by the reactions of the control. It goes without saying, that the constancy of the receptor strength had been established by earlier experiments. The results of the titrations within the family material are depicted in figure 1.

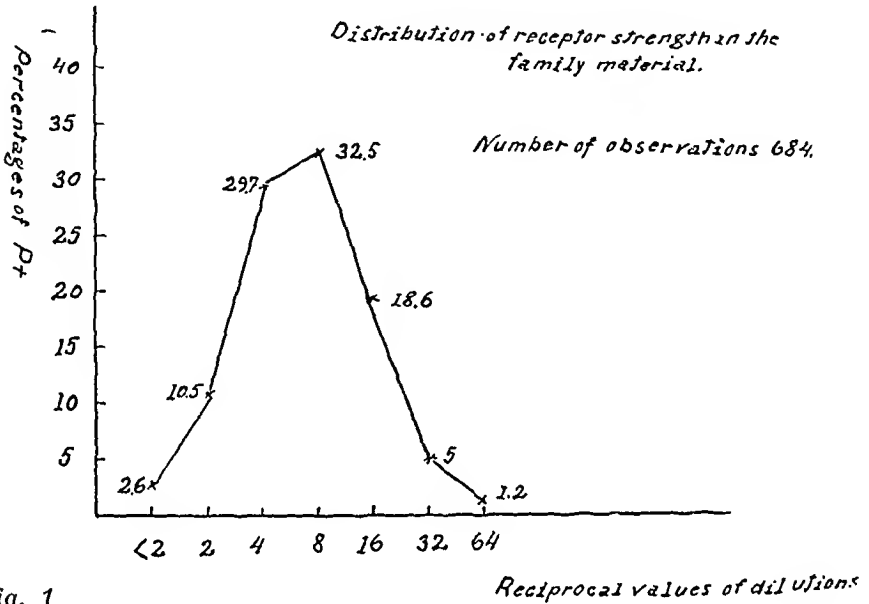


Fig. 1

Just as in the formerly published, unselected material (8) no indication is found of distinct classes according to strength. In order to investigate, if a possible masking of such classes existed due to greater receptor strength in homozygous than in heterozygous bloods, the results of the titrations of all known heterozygous samples from the family material are depicted in figure 2. It is seen, that even if the contour of the curve has changed considerably, still no distinct classes may be discerned.

On the other hand it appears from the pedigrees of the families, that the receptor strength to a great extent depends on homo- and heterozygosity, but further that the variations without any doubt are hereditary properties, and that a given strength, if not modified by homozygosity, is inherited without significant change. However, in order to analyse the mechanism of inheritance it is convenient to divide the material into several classes according to strength. Assuming a difference in receptor strength of two titration steps as significant, a classification is chosen, by which each class comprises two titer values with the exception of the strongest class, which comprises three

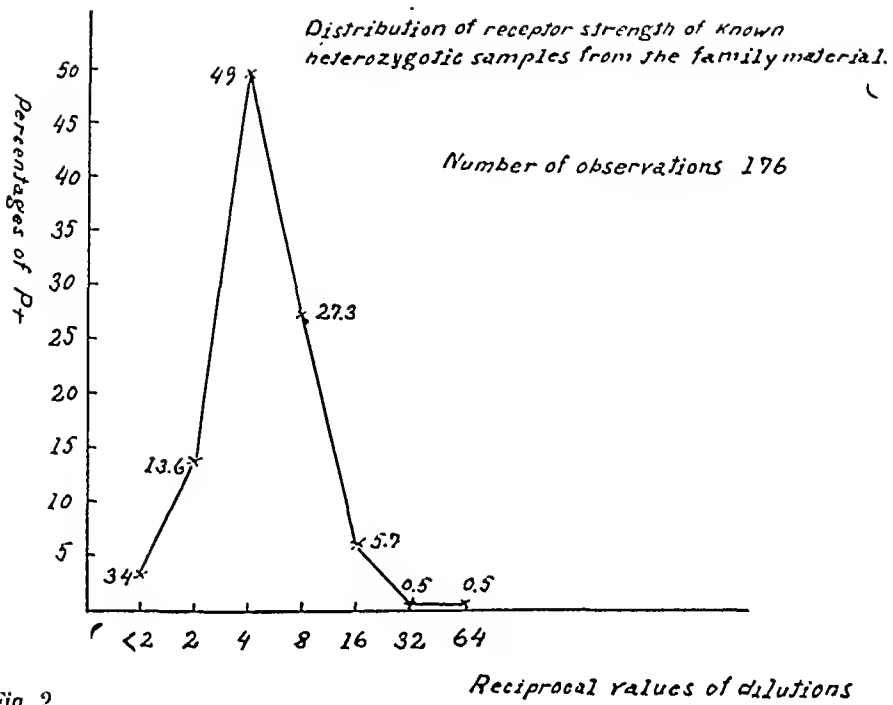


Fig. 2

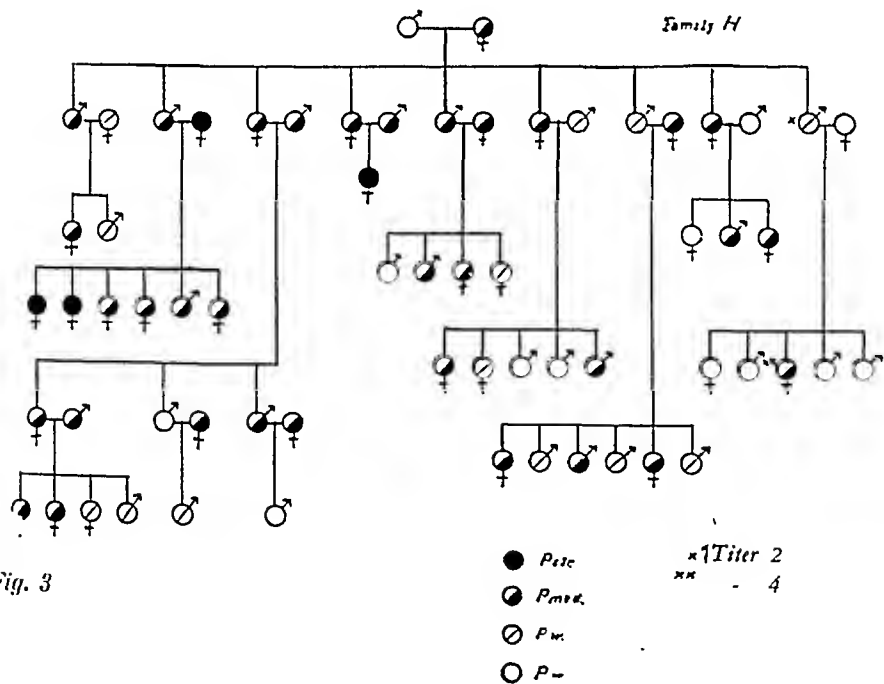


Fig. 3

in consideration of the small number of bloods of the highest titration values. In classification the limits between classes have to be sharp, but it must be emphasised, that the actual titration curve exhibits no distinct classes, and that due to experimental hazards specimens of a receptor strength in the vicinity of the selected limits may just

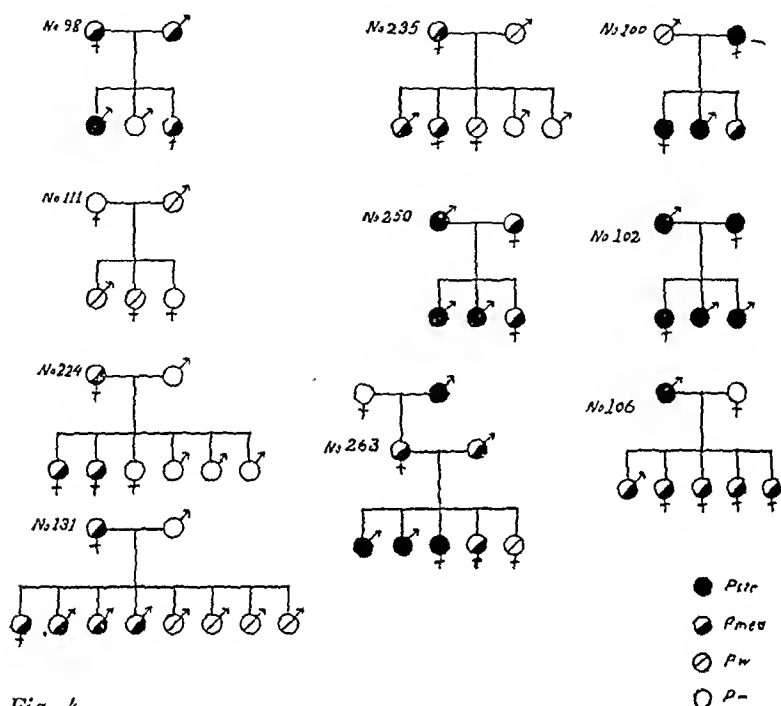


Fig. 4

as well be enlisted above as below the limit. Arbitrarily 3 classes of phenotypes are chosen, viz. *P*strong for receptor strength of titer 16 and more than 16, *P*medium for titer 4 and 8, and *P*weak for titer 2 and less than 2. Using this classification in the pedigrees of the families it is evident, that the receptor strength is due both to different genes conditioning to different strength and to homo- and heterozygosity. Consequently the gene *P'* cannot be an entity, but has to be interpreted as a group of allelic genes, all of which dominate over *p'*. In figures 3 and 4 an example is given of the pedigree of a complete family, and further several pedigrees are selected to illustrate the complex origin of the phenotypical receptor strength.

A total of 183 matings with 508 children has been classified in this way. The various combinations of matings and the proportions of their offspring are shown in table 5.

Table 5.

Matings	Number	Offspring			
		Pstr.	Pmed.	Pw.	P—
1. Pstr. — Pstr.	13	24	8	0	7
2. Pstr. — Pmed.	36	33	60	4	2
3. Pstr. — Pw.	6	5	6	2	0
4. Pstr. — P—	20	0	39	5	5
5. Pmed. — Pmed.	46	17	84	13	19
6. Pmed. — Pw.	18	4	32	11	5
7. Pmed. — P—	34	0	48	17	30
8. Pw. — Pw.	2	0	3	2	1
9. Pw. — P—	8	0	4	6	12
Total	183	83	284	60	81

The distribution of 183 matings with 508 children using the proposed classification according to receptor strength.

From this table several interesting facts are seen. Primarily it is obvious, that the variation in receptor strength cannot solely be due to homo- and heterozygosity with regard to a single gene conditioning to one single receptor strength. The presence of P— children from the matings Pstr. — Pstr., which in accordance with such a hypothesis

Titration values in families exhibiting discrepancies to the proposed theory

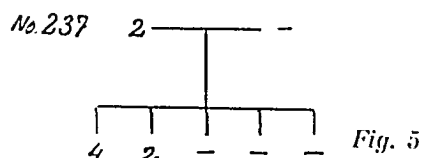
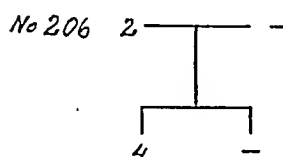
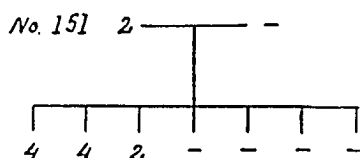


Fig. 5

ought to be homozygotic, strongly tells against this. On the other hand the greater percentage of heterozygosity among the classes of lesser receptor strength is clearly indicated by the greater proportion of P — children in those classes. In regarding group 9 of the matings it appears that 4 children have a receptor strength greater than that of the positive parent. Two of these children originate from mating no. 151, the others from matings no. 206 and 237. From the pedigrees in fig. 5 it is revealed that the difference between the receptor strength in parent and offspring in those cases is one titration step and consequently cannot be considered significant.

Thus the following general rule appears to hold true, that offspring from P + — P — matings cannot have a receptor strength of greater potency than that of the positive parent, provided that a difference of at least two titration steps between the receptor strength of parent and offspring is claimed for the difference to be significant.

On the other hand it is seen, that two parents of weak or medium strength may have offspring of medium respectively strong receptor strength indicating the greater strength of homozygotic versus heterozygotic bloods.

It thus appears, that the receptor strength is conditioned genetically, and that the strength conditioned by the various genes is a constant, hereditary property. The fact, that the observed phaenotypical differences are quantitative and not qualitative, does not necessarily exclude the possibility of different allelic genes, and the absence of sharply differentiated classes in heterozygotic individuals may well be due to the coarseness of the method of investigation, but on the other hand it may be due to a multiplicity of closely related genes in conformity with the findings regarding D and D^u within the Rhesus blood group system. (13).

Assuming the phaenotypical classification mentioned above, a theory of four allelic genes agrees closely with the results observed. Designating the genes p, q, r and s for P —, Pw, Pmed. and Pstr. respectively, 10 genotypes are possible ss, sr, sq, sp, rr, rq, rp, qq, qp, and pp. From the pedigrees and table 5 the genotypical composition of the various phaenotypes is deduced and given in table 6.

Table 6.

Phaenotype	Genotype
Pstr.	ss, sr, sq, sp, rr.
Pmed.	rq, rp, qq.
Pw.	qp.
P—	pp.

The genotypical combinations of the various classes of factor P.

Knowing the proportion of the different phaenotypes in a population the frequencies of the corresponding genes are easily calculated. The distribution of the phaenotypes among parents and offspring in the family material is given in table 7.

Table 7.

	Pstr.	Pmed.	Pw.	P—	Total
Parents	24 (88)	48 (180)	10 (36)	18 (66)	100 (370)
Offspring	16 (83)	56 (286)	12 (60)	16 (81)	100 (510)

The proportion of the various classes in parents and offspring in percentages. In parenthesis the actual number of observations.

As the receptor strength apparently is not fully developed in the offspring, the gene frequencies are calculated from the figures of the parents.

$$p^2 = 0.18, p = 0.425$$

$$2qp = 0.10, q = 0.118$$

$$q^2 + 2rp + 2rq = 0.48, r = 0.429$$

$$p + q + r + s = 1, s = 0.028.$$

These frequencies agree very well with the corresponding frequencies as read from figure 2. From this curve the frequencies of the assumed genes may be read directly, if the percentages plotted on the curve are multiplied with $1-p$. As $1-p$ approximately amounts to 0.6 the frequencies of the genes are:

$$q = 0.17 \times 0.6 = 0.102$$

$$r = 0.76 \times 0.6 = 0.455$$

$$s = 0.067 \times 0.6 = 0.04$$

It is now possible to calculate the phaenotypical distribution of the offspring from the various combinations of matings. From table 5 it is seen, that combinations no. 2, 5 and 7 are the most frequent and represent a relatively large number of children, and consequently those groups of matings are chosen to test the theory. Prior to the calculation still another thing must be taken into consideration, viz. the difference in receptor strength in parents and offspring. It appears from table 7, that there is a lesser percentage Pstr. among the offspring than among the parents, and that a correspondingly greater percentage of Pmed. is found in the offspring, whereas the percentages of Pw and P— do not differ significantly. Even if the difference between the percentages of Pstr. and Pmed. in parents and offspring does not exceed 3 times the standard error, it may be considered significant in view of the statements, reported earlier, of the weaker development of receptor strength in children. (8). It is seen, that within class Pstr.

the proportion of the observed frequencies in parents and offspring is 3 : 2. It appears justifiable to apply this correction to the figures of the offspring in the observed matings, thus multiplying the observed frequency of Pstr. children with $3/2$ and subtracting the corresponding number from the children of phaenotype Pmed. In table 8 the observed and the calculated figures are given.

Table 8.

Matings	Offspring			
	Pstr.	Pmed.	Pw.	P—
Pstr. — Pmed.	50.1 (51.5)	43.9 (45.4)	4 (1.2)	2 (1.9)
Pmed. — Pmed.	19.2 (23.5)	56.8 (52)	9.7 (10)	14.3 (14.5)
Pmed. — P—	0 (0)	50 (48.5)	18 (13.5)	32 (38)

The distribution of offspring from different matings according to receptor strength. The figures are percentages after correction. The calculated percentages are given in parenthesis. The actual number of matings and offspring appears from Table 5.

The agreement between the observed and the calculated figures appears fairly good and may well be taken as proof of a mechanism of heredity governed by multiple allelic genes, one of which, *p*, conditioning to the absence of the *P* factor, and a group of genes, *P'*, conditioning to different receptor strengths. The genes for the presence of the *P* factor dominate over *p*, and further homozygous receptors phaenotypically are of greater strength than heterozygous, provided that the genes in question condition to the same receptor strength. The classification of *P* + into 3 classes, which appears reasonable, necessitates the presence of at least 3 genes for the *P* factor, in view of the fact, that some of the bloods in the phaenotypical class Pstr. are heterozygotic. Further the variation of strength of the heterozygotic bloods ranging from titration steps of less than 2 to 64 indicates at least 3 genes, if a difference of two titration steps is considered significant. The presence of 3 allelic genes conditioning to the *P* factor in connection with the augmented receptor strength in homozygosity may well explain the variation of phaenotypical receptor strength, but on the other hand the possibility of more than 3, closely related genes cannot be excluded and may be very probable, but the coarseness of the technical method together with the fact, that the various genotypical properties condition to quantitative differences prohibit a more exact analysis of the problem.

However, the possibility of a multiplicity of genes does not in any way affect the considerations on the principles of the inheritance mentioned above.

The entire family material has been analysed with regard to ex-

ceptions to the proposed theory of inheritance. Disregarding exceptions due to a difference of one titration step a couple of exceptions remain, and these may well be explained by a retarded development of the receptor strength in the children.

From the present material it thus appears, that the proposed theory holds true for practical purposes.

The medico-legal application of blood factor P.

The medico-legal application of blood factor P is closely related to its heredity, as the chief purpose of P grouping is the exclusion of paternity in cases of disputed parentage.

1933 *Schiff* in a paper on the forensic use of blood types M and N mentioned the possibility of using factor P in medico-legal cases but concluded, that the heredity had not yet been adequately investigated for that purpose. (15).

1941 *Dahr* presented a series of paternity cases, in which P grouping was employed resulting in a few exclusions. In his conclusions *Dahr* states, that the law of heredity of factor P has not been established with certainty for legal purposes, but in all probability the assumed theory is correct, and incompatibility with this tells strongly against the paternity of the man in question. (6).

Jungmichel (10) agrees with the statement of *Dahr*, while *Andresen* is inclined to modify the statement a little and prefers considering P exclusions along with other evidence of the nonpaternity of the accused man. (1, 2).

Wolff and *Jonsson* state, that a typical exclusion within the P system with a moral certainty tells against the paternity of the alleged putative father. (17, 9).

When considering the presence or absence of factor P alone, only one possibility of exclusion is present viz. the mother being P —, the child P + and the accused man P —.

The reliability of this exclusion depends on several circumstances. Primarily the correctness of the assumed law of inheritance is of supreme importance. The material supporting this theory has gradually accumulated, and in a biological sense the law of inheritance is quite certain. In table 9 are presented the results of the family investigations published till now. (From the material of *Landsteiner* only 4 P — — P — matings are included in the table).

Further a total of 1201 mother-child examinations (*Wolff*, *Jonsson*, present author) and 383 examinations of twins (*Dahr*) strongly supports the assumed theory.

The five apparent exceptions to the theory encountered in table 9 may well be ascribed to illegitimacy as mentioned above.

Table 9.

Matings	Investigators	Number of matings	Offspring	
			P+	P-
P+ — P+	Dahr	319	980	131
	Jungmichel	25	80	11
	Sanger et al.	49	99	11
	present author	180	430	49
P+ — P—	Dahr	194	505	277
	Jungmichel	13	32	21
	Sanger et al.	34	53	21
	present author	86	150	62
P— — P—	Dahr	50	4	173
	Jungmichel	2		7
	Landsteiner	4		18
	Sanger et al.	2		6
	present author	15	1	35
	Total	888	2334	822
			3150	

The results of family investigations published till now with the exception of most of the families of Landsteiner and Levine.

On the other hand the application of a biological law to legal cases demands an exceedingly vast amount of experience in order to ascertain, that exceptions to the law do not occur. In this respect especially the matings P — — P — are of interest this combination being the only one, which may disclose contingent exceptions. As so-called impossible mother-child combinations as present within the ABO- and MN systems, for instance mother O, child AB etc., do not occur within the P system (at any rate not till a possible agglutinin against P — may be encountered) the significance of matings P — — P — is still greater. Disregarding the 5 apparent exceptions as due to illegitimacy the remaining 239 P — children indicate that true exceptions to the assumed law of inheritance are not encountered. Even if the number of observations is still limited, and consequently the correctness of the theory is less certain than for instance the laws governing the ABO and MN inheritance, it is considered justifiable to state the paternity of an accused man as exceedingly improbable, if it is incompatible with the assumed law of inheritance of factor P. The presupposition of such a statement is of course a reliable technic in testing the bloods, i. e. the examination has to be done by a »blood grouping expert« (16), familiar with P grouping, using both animal and human sera and checking the agglutination results by absorption experiments. The fact, that nevertheless a few children of weak, not

fully developed receptor strength may falsely be designated P —, does not involve any false exclusions of paternity, but just fails to disclose an actual exclusion in the case in question.

With regard to the inheritance of receptor strength several possibilities of exclusion of parentage are present as demonstrated by the family material of this investigation. For practical purposes, however, »exclusions« have to be limited to the matings P + — P —. Offspring from these matings cannot have a receptor strength greater than that of the P + parent. In order to obtain reliable results for medico-legal purposes it is necessary to perform the titrations of all bloods from the case in question at the same time, using the same serum and including bloods of known strength in the set up. Further the bloods have to be quite fresh and preferably all drawn simultaneously, and finally the difference in strength must exceed at least two titration steps to be significant. Preferably the result may then be used as evidence in connection with other circumstances telling against the paternity of the accused man.

Disregarding the exclusions based on differences in receptor strength, the theoretical percentage of exclusions by employing P grouping in paternity cases is 2.4 per cent. The theoretical percentage of exclusions in Denmark using the A₁A₂BO- and MN-systems is 32.5 per cent. Subtracting exclusions by more than one system the employment of the P system is going to augment the percentage to 34.1 per cent. In Denmark the paternity cases, all of which are tested at the University Institute of Legal Medicine, amount to about 2000 a year. As the actual percentage of exclusions is about 50 per cent of the theoretical, exclusions due to P grouping shall probably occur in about 16 cases a year. Considering the serious economic and other consequences, which result from a verdict pronouncing a man the father of the child of another man, even this small number of exclusions may well justify the inclusion of P grouping into the routine examinations of bloods from paternity cases.

Summary.

- 1) After a brief review of the investigations published earlier on the heredity of blood factor P, the author's material is presented. It comprises 700 mother-child determinations and a family material of 54 families comprising 281 matings with 727 children.
- 2) By analysis of the material the results conform with the formerly assumed theory, by which the inheritance of factor P is governed by 2 allelic genes P' and p', the former dominating over the latter. In consequence of this *the first rule of heredity* is deducted: a child cannot have blood factor P, unless this is present in the blood of at least one of the parents.

- 3) By titrations of the receptor strength within the family material it is evident, that the strength is a hereditary property too, and evidence is found, that the strength is transmitted through several, closely related, allelic genes, all of which dominate over the gene conditioning to the absence of the P factor. The exact number of genes for the P factor cannot be estimated, but the variation in phaenotypical receptor strength cannot be explained by less than 3 positive genes, even if the greater receptor strength found in homozygotic bloods tends to a dispersal of the phaenotypical receptor strength. The fact that gene P' is not an entity but a group of genes does not affect the validity of the first rule of heredity, as gene p' is dominated by all positive genes, and as the differences conditioned by these genes are quantitative not qualitative.

According to these considerations a *second rule of heredity* is proposed: offspring from the mating of a P positive and P negative parent cannot have a receptor strength greater than that of the P positive parent.

- 4) The medico-legal application of blood factor P as applied to cases of disputed parentage is discussed. With regard to exclusions according to the first rule of heredity the statement »exceedingly improbable« is proposed for contradictions to this rule. Contradictions to the second rule, however, are as yet less reliable as a means of exclusion, and may preferably be considered together with other circumstances telling against the paternity of the accused man. The theoretical percentage of exclusions by contradiction to the first rule is calculated to be 2.4 per cent, and the actual percentage of exclusions in Denmark by employing P-grouping alongside the A₁A₂BO- and MN-systems is found to be about 0.8 per cent i. e. about 16 exclusions a year.

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SPERMATIC INVASION OF THE EPIDIDYMIS

By *Sten Cronqvist.*

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Judging by the scantiness of the reports available on sperm invasion of the interstitial tissue in the epididymis, it would seem as if this affection has been given but relatively little attention. The condition was first mentioned in 1898 by *Beneke* in the course of a discussion after a lecture by *Simmonds*. In 1922 *Cunningham & Cook* published an article in *Journal of Urology* and showed that the contents of an acutely inflamed ruptured ductule will be extruded into the stroma and that the consequential abscesses will contain spermatozoa. Two cases of epididymitis with sperm invasion were described in 1924 and 1940, respectively, by *Orsos*, who, for the first time pointed out the possibility of sperm invasion being a cause of epididymitis. and in 1932 in the section on diseases of the male reproductive system in *Hencke-Lubarch's Manual* *Oberndorfer* described a similar case with a history of previous epididymitis. In *Steinberg & Straus's* case of sperm invasion in a 32-year-old man reported in 1947, there was likewise a previous history of epididymitis. *Oberndorfer* and *Simmonds*, however, claim that they have often observed sperm invasion and, judging by our experience we are inclined to the view that the affection is much more common than might be inferred from the literature.

A search of the records of the epididymis material of a five-year period, 1944—48, at the Institute revealed the presence of spermatozoa in the interstitial tissue to be no rare by-finding in cases of non-specific epididymitis. In addition hereto 7 cases were seen in which the sperm invasion dominated the picture and in which the inflammation was confined mainly to the area around the invading spermatozoa. These cases will now be reported.

Case Reports.

Case 1. A. T. 30 yrs. (J. Nr. 2006/48, Surg. Dept. Lund). In 1944 pat. had been admitted for: Status post. urethrit. subsechron. + prostatitis et vesiculit. chron. + epididymitis chron. Treated in 1947 for: Abscessus prostatae (tuber-

culosis?). April 1948: Left epididymis was swollen, indurated and nodular; it was not tender. There was neither prostatic abscess nor evidence suggestive of tuberculosis. Epididymectomy was done on 20th May 1948. The resected epididymis exhibited a fibrous, granular parenchyma. The tail was studded with small hard nodules.

Microscopic Appearance. Moderately increased connective tissue. The ductules were somewhat dilated, in some instances the dilatation was marked,



Fig. 1.

Local destruction of the tubular wall with the escape of spermatozoa and lively reaction of the tissue (Case 1).

and then the epithelium was low and flattened. In the increased connective tissue there was a moderate, diffuse, and preponderantly lymphocytic infiltration as well as ragged-shaped foci. These foci were built up of edematous connective tissue and of epithelioid and inflammatory cells mixed with spermatozoa. Neither necrosis nor giant cells were seen. In some areas of the ductules the epithelium had undergone destruction and exhibited perforations through which the ductular contents were able to escape into the stroma. There was thus intercommunication between the ductule and the inflammatory focus (Fig. 1). In addition hereto many of the lymph vessels were filled with sperm (Fig. 4).

Case 2. N. O. R. 40 yrs. (J. Nr. 1863/46. Hörby Hosp.). 1944: non-specific urethritis. June 1946: acute left epididymitis which passed into a chronic state with persistent enlargement and pain. December 1946: epididymectomy. Epididymis: swollen and nodular with a pea-sized abscess near the border of the testis.

Microscopic Appearance: Increased interstitial tissue with sparse inflam-

matory cellular infiltrates. The ductules were dilated and contained an abundance of sperm. It was observed that in a few scattered areas through a partially destructed epithelium the spermatozoa had been forced out into the surrounding tissue where they were surrounded by a lively inflammation with giant cells and a number of epithelioid cells. Several granuloma-like foci were likewise observed. The foci were built up of edematous connective tissue and epithelioid and inflammatory cells with spermatozoa. Accumulations of spermatozoa were found also in the proximate lymph vessels.



Fig. 2.

»Foreign-body-granuloma« in the interstitial tissue (Case 7).

Case 3. M. P. 32 yrs. (J. Nr. 435/48. Landskrona Hosp.). Since 1936 pains in the back and down along the groins. There was no discomfort from micturition but occasional acute pains and a sensation of burning. Considerable loss of weight. Acid-fast bacilli were found in the urine. Guinea-pig test and culture gave negative results. X-ray: no evidence of tuberculosis. The left epididymis, however, slightly enlarged and tender. Epididymectomy was done on 26th Feb. 1946.

Microscopic Appearance. No increase of the interstitial connective tissue. The ductules were dilated considerably and in some cases contained enormous accumulations of spermatozoa, occasionally also fibrin and inflammatory cells. The epithelium was as a rule low and flat. In some of the tubules the wall had undergone destruction and the contents been forced into the surrounding stroma where they were surrounded by lively »foreign-body-reaction«.

Scattered deposits of iron pigment seen in the interstitial tissue indicated an earlier trauma.

Case 4. S. E. G. 50 yrs. (J. Nr. 2685/48. Norrköping Hosp.). 1941: cystitic symptoms and later that year right epididymitis. Dec. 1948: swollen testicle and right epididymitis. No trauma. No urethral discharge. Epididymis was hard, nodular and tender. Testis: smooth, tense and elastic, vas deferens was somewhat thicker than a lead-pencil. Epididymectomy was done on 7th Jan. 1949. The gross appearance of the resected epididymis resembled that seen in chronic epididymitis.

Microscopic Appearance: The epididymis was very fibrous and showed

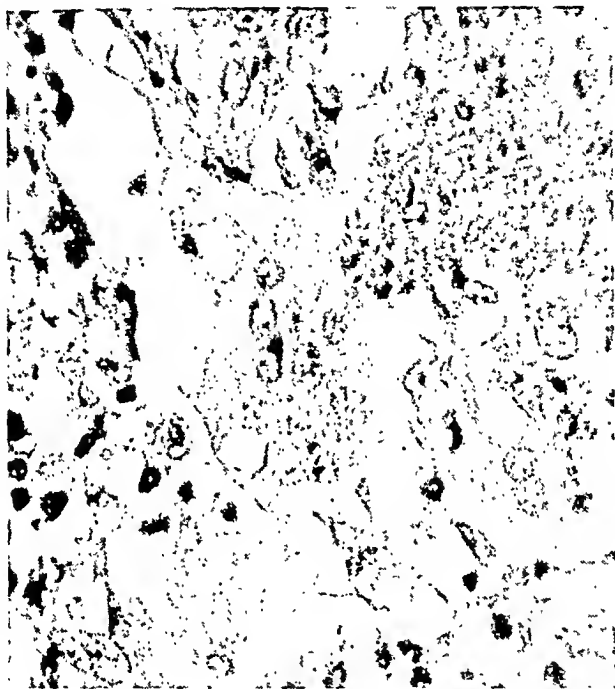


Fig. 3.

Enlargement of previous picture. Some spermatozoa are seen intercellularly, others in a giant cell.

moderate cellular infiltration. Scattered granulation foci containing an abundance of spermatozoa and epithelioid cells were likewise observed. The ductules were dilated but in most cases the epithelium was preserved. The epithelium had in one or two areas undergone destruction; in these areas the ductular epithelium had been replaced by a pale granulation tissue with rich accumulations of spermatozoa. Distally to this destruction there was evidence of a more marked inflammation with stroma loosened by edema and infiltrated with round cells and numerous leukocytes.

Case 5. B. J. 27 yrs. (J. Nr. 41/46 Eksjö Hosp.). 1942: Admitted for: Epididymitis dx. No evidence of tuberculosis. October 1945: recurrent right epididymitis. On admittance in Jan. 1946 the right testis and epididymis were slightly swollen and somewhat tender. Epididymectomy was done on 11th Jan. 1946. The resected epididymis was possibly slightly enlarged and somewhat fibrous but no clear-cut macroscopic evidence of pathologic changes were to be seen.

Microscopic Appearance. The epididymis was moderately fibrous. The in-

terstitial tissue was increased and in some areas there were rich accumulations of diffuse cellular infiltrates, whilst in other areas the infiltration was rather scanty. Scattered areas of the connected tissue had been loosened by edema and these areas had been infiltrated by spermatozoa and round cells. Most of the ductules of the epididymis seemed somewhat dilated, and a few of them contained traces of pus and of sperm.

Case 6. G. J. 32 yrs. (J. Nr. 2213/46 Karlskrona Hosp.). May 1946 treated for acute non-specific right epididymitis. One week after discharge the symp-

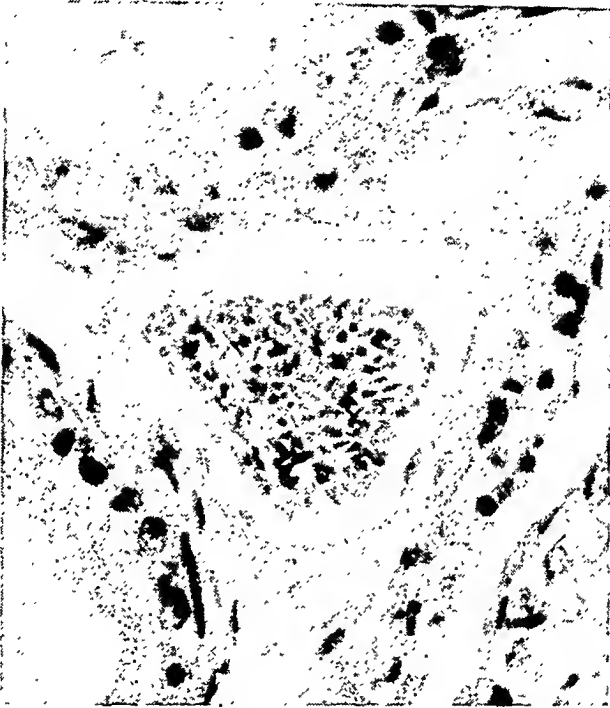


Fig. 4.

Accumulation of spermatozoa in a lymph vessel
(Case 1).

toms returned. The tail of the right epididymis, which was rather firm and nodular, had swollen to the size of a large walnut. There was no tenderness, neither were there signs of tuberculosis. Epididymectomy was done on the 12th June, 1946. The operation specimens showed macroscopic changes in the tail only.

Microscopic Appearance. The stroma of the epididymis was very fibrous. Some of the ductules had been destroyed by fibrous granulation tissue, whilst others were to a varying degree obliterated by the proliferating epithelium. The tissue, extensively infiltrated by lymphocytes and leucocytes, displayed isolated rounded foci in whose centres the stroma had been loosened and had been the site of histiocytic elements, lymphocytes, and leucocytes, as well as abundant spermatozoa which were also on their way out into the surroundings of the granuloma.

Case 7. G. S. 53 yrs. (J. Nr. 2161/48 Surg. Dept. Hälsingborg Hosp.). In December 1948 cystic catarrh with high fever followed by a swelling and reddening of the left half of the scrotum with pain radiating along the spermatic cord. No urethral discharge. Local condition on admittance: left half

of scrotum swollen. The skin of the scrotum was loose. Left epididymis was abnormally thick; the spermatic cord was also swollen. After the acute inflammation had subsided there persisted a hard tender swelling of the epididymis. Examination disclosed no evidence of tuberculosis. Epididymectomy was done on the 6th Sept. The cut surface of the resected epididymis showed no definite macroscopic changes.

Microscopic Appearance: Epididymis had been the site of marked inflammatory changes. The ductules were to a varying degree dilated. Some of them were lined with what seemed to be a stratified squamous epithelium, others by practically normal epithelium. They contained retained secretion and an abundance of spermatozoa. The interductular tissue was increased and fibrous and showed abundant lymphocytes and leukocytes as well as granuloma-like foci in whose centres were lodged spermatozoa and some eosinophiles, leucocytes and large epithelioid elements. In the periphery, were a number of giant cells containing in their cytoplasm spermatozoa — partly apparently preserved, partly destroyed. Isolated accumulations of spermatozoa were seen on their way out to the surrounding tissue (Fig. 2 and 3).

A report is given of 7 cases of sperm invasion of the interstitial tissue of the epididymis. Common to all is primarily the fact that the inflammatory changes are most marked and are mainly confined to the site of the spermatozoa in the interstitial tissue, where they form granuloma-like foci (see Fig. 2), and secondly, that the ductules of the epididymis are dilated and filled with sperm (except in Case 6). In the first four cases the granuloma-like foci are in communication with the ductules via a lesion in the wall of the latter (see Fig. 1). Five of the cases had a history with a previous epididymitis, in four of them more than 1 year before, and in one case, only 1 week before the examinations in question. In another case the patient had previously had cystitis, whilst in the remaining case the presence of iron pigment was suggestive of earlier trauma.

Discussion.

As mentioned in the introduction, the occurrence of sperm in the interstitial connective tissue is not an infrequent by-finding in acute epididymitis. Microscopic examination of the resected epididymis often shows an increased interstitial connective tissue infiltrated with lymphocytes, leucocytes, and plasma cells. The tubules have generally been transformed into abscesses containing inflammatory cells, shed epithelial cells, fibrin and sometimes a varying amount of sperm. The epithelium lining the tubule had frequently undergone total destruction and one sees how the contents are forced out into the surrounding stroma and even into the lymph vessels. In all of these cases it seems obvious that the widespread inflammatory changes have caused lesions of the walls of the ductules and thereby allowed the spermatozoa to escape into the interductular tissue.

In the 7 cases described above, however, the inflammatory changes

seen in the resected epididymis are essentially confined to the site of the sperm invasion, and the question whether the intruding sperm are responsible for the inflammatory reaction seems to be motivated. In these cases one might imagine an inflammatory reaction directed against a physiological substance analogous to the »foreign-body-reaction« against urine (*Helmke*) and mucus (*Hamperl*).

Steinberg & Straus envisage three causes of spermatie invasion:

1. Active spermatie invasion through intact ductular epithelium,
2. Focal inflammation with ulceration of the epithelium,
3. Degeneration and atrophy of the ductular epithelium.

Orsos believes the first possibility to be responsible for the affection in his two cases, in which an inquiry into their histories disclosed no evidence of an earlier inflammatory affection of the reproductive system. He bases his opinion on his own observation of the site of the sperm — isolated spermatozoa at a considerable distance from the foci proper some even lodged in connective tissue cells — and partly on the fact that he was unable to detect any epithelial rupture to permit the escape of the sperm, and partly on *Kowalewsky's* observations, according to which spermatozoa are able to penetrate different tissues of the leech. The ability of the sperm to penetrate intact epithelium cannot, of course, be excluded, but *Lehner's* observation that an intact membrana propria arrests the further migration or penetration of sperm argues against this possibility. Moreover, it should be borne in mind that a possible focal destruction of the epithelium is very quickly repaired thanks to the extraordinary regeneration capacity of this tissue (*Oberndorfer; Kyrle & Schopper*). A defect occurring in the epithelium simultaneously with the onset of the inflammation and permitting the escape of sperm would most probably have healed by the time of the operation so that, as far as the epithelium is concerned, the microscopic picture of the resected epididymis would be normal.

As to the second possibility, focal inflammation with ulceration of the epithelium, acute inflammation is undoubtedly able to lead to a destruction of the epithelium and the ductular wall and thus permit the escape of the spermatozoa into the surrounding tissue (*Cunningham & Cook*). This is what happened in the cases briefly referred to in the introduction in which the spermatie invasion was observed as a by-finding in acute epididymitis, and also in *Oberndorfer's* case of subsiding epididymitis, in which he saw spermatozoa in the stroma outside the more or less damaged ductules. *Steinberg & Straus* believe that the history of a previous acute epididymitis in both *Oberndorfer's* case and their own argue for the assumption that sperm invasion had occurred via a focal destruction of the tubular wall caused by the earlier inflammation. Two of the four cases reported earlier in the literature and two of my own in which there was no previous history of epididymitis, as well as *Steinberg & Straus's* cases and four of my

tory changes around the sperm all cases showed dilated tubules with flattened epithelium and signs of stagnation. In four of the cases there was direct communication between the granuloma-like foci and the ductules. There was a history of a previous epididymitis in five of these seven cases.

In the discussion it is pointed out that the localization of the inflammation around the invading sperm motivates the question whether the inflammation is to be ascribed to the intruding sperm — »foreign-body-reaction«.

Possible causes of spermatic invasion are also discussed. It is believed that the most probable explanation of the condition is an increased intratubular pressure which, judging by the microscopic pictures, was present in six of the author's cases and in 2 of the four cases reported in the literature. It is possible that the increased pressure alone might be sufficient to rupture the wall, but it is held more probable that the rupture is due to the simultaneous effect of increased intratubular pressure and locally decreased resistance of the wall of the tubules due to an earlier or active inflammation.

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SKIN TUMOURS PRODUCED IN MICE BY CARCINOGENIC HYDROCARBONS SOLUBILIZED IN AQUEOUS SOLUTIONS OF ASSOCIATION COLLOIDS. I.

By *Per Ekwall* and *Kai Setälä*.

(Received for publication February 14th, 1949.)

In a previous paper concerning the solubilization of certain carcinogenic hydrocarbons by association colloids, *Ekwall*¹ has observed that above their so-called critical concentration for micelle formation, association colloids of different types have the ability to bring carcinogenic hydrocarbons into aqueous solution. Further, it was found that the solutions obtained were clear, absolutely homogenous and stable. The solubility of hydrocarbons increased in some cases in the same proportion as the concentration of the colloid, and in other cases as the square of the colloid concentration^{1, 2, 3}. It was also found that when painted on the skin of mice the carcinogen was rapidly assimilated and the fluorescence microscope revealed that the carcinogen (changed and/or unchanged) had accumulated in the same places as when applied by painting with an acetone solution of the hydrocarbon³.

Carcinogenic hydrocarbons are fat-soluble and practically insoluble in water. Fat-solvents (benzene, acetone, chloroform, *etc.*) have therefore been used as carriers and spreaders of these substances⁵. For subcutaneous, intravenous or oral administration they are, however, out of the question. Also, certain experiments, *e. g.*, investigations with added substances («cocarcinogenesis») which are not always fat-soluble, experiments concerning the elimination of the carcinogen, but especially the study of certain surface-chemical properties of the carcinogens as well as numerous chemical and biological aspects, cannot be carried out in such fat dissolving (often toxic) media.

Therefore, numerous attempts have been made to circumvent this limitation of insolubility in water. None of the methods reported in the

literature have been ideal for all purposes: some of the »solvents« have been toxic, the concentration of the carcinogen has been too low, the suspended particles coarse, the solutions have not been stable, etc. It may be mentioned especially in this connection that *Weil-Malherbe* introduced the 1.3.7.9-tetramethyluric acid as a solubilizer for benzpyrene^{10,17}. He gave two series of mice a single injection of 0.3 c. c. of a 5 per cent aqueous solution of tetramethyluric acid saturated with benzpyrene (about 0.2 mg per mouse). Among 21 mice alive after 8 months only one sarcoma arose locally (5 months after injection). *Gummel* and *Rare*¹⁸ used sodium cholestenone sulphonate solutions as carriers for benzpyrene. After 5 months they found degenerative changes in parenchymatous organs but no tumours. *Fieser* and *Newman*⁴, and *Shear* and *collab.*^{12,13} have studied the carcinogenic potency of the water-soluble sodium salts of chloric acids of desoxycholic acid with carcinogenic hydrocarbons. They obtained tumours when the solutions were subcutaneously administered. *Stamer*¹⁴ has prepared suspensions of a synthetic compound called »Postonal« (a derivative of polyethylene oxide). The suspension was, however, unstable and it had to be employed within one hour. *Setälä*^{9,10,11} has used certain water-soluble polyethylene glycols (both liquid and solid) as carriers for some carcinogenic hydrocarbons, and has observed that when e. g., the solid »Carbowax« (trade mark for certain polyethylene glycols) was used as spreader for the carcinogen, the hydrocarbons presumably permeate easily into the tissues. It was also found that treatment of the mouse skin with carcinogenic hydrocarbons dissolved in these compounds resulted in multiple, progressively growing tumours.

As mentioned above, *Ekwall* and *Setälä*³ have found that aqueous solutions of association colloids, such as sodium myristate, oleate, myristyl sulphate, cholate, »Triton NE«, etc. are able to solubilize the carcinogenic hydrocarbons. Experiments concerning the activity of the carcinogenic hydrocarbons solubilized by different association colloids are now in progress. The present paper deals with the production of cutaneous tumours in mice treated with 9.10-dimethyl-1.2-benzanthracene solubilized in aqueous sodium cholate solutions.

Material and Methods.

Experimental animals. — The mice — about two months old at the beginning of the experimental series — were of a mixed strain. The animals were divided into subgroups, 10 mice in each.

The mice were painted three times weekly on a large area of the back for a definite period after which all the animals were killed on the same day.

Incidence of tumours. — This was estimated as mentioned in our previous communications.

The carcinogenic compound. — As carcinogen 9.10-dimethyl-1.2-

benzanthracene (Eastman Kodak Company, Rochester, N. Y.) was used.

Solvents. — The sodium cholate solutions were prepared in the following manner: cholic acid (Hoffmann-La Roche, Basle) was dissolved in the smallest possible amount of absolute ethyl alcohol by warming, and then neutralized with an equivalent amount of sodium ethylate in absolute alcohol. A part of the alcohol was removed by distillation and the sodium cholate which crystallized out on cooling was filtered and dried. Aqueous solutions of the salt were prepared by dissolving in conductivity water. Twenty per cent and eight per cent aqueous sodium cholate solutions were shaken with finely powdered 9.10-dimethyl-1.2-benzanthracene in a thermostat at 40° C until the solutions were saturated with the hydrocarbon. Excess hydrocarbon was separated by filtration, after which the solutions were diluted with the corresponding pure sodium cholate solutions to prevent the separation of the hydrocarbon by crystallization owing to the cooling to room temperature or the slow evaporation of water. The strongly fluorescent solutions of aqueous sodium cholate contained 2500 mg. carcinogen/1000 c. c. in 20 per cent solution.

For control purposes the following solvents were used: reagent grade acetone, anhydrous lanolin, solid »Carbowax 1500«, and dioxane. In liquid solutions of the control series the concentration of the carcinogen used was 0.03 to 0.3 per cent. and in the solid solvents 0.25 per cent. respectively.

Results.

Incidence of tumours (Fig. 1).

Sodium cholate as carrier for the carcinogen. — By the 4th week of the experiments (i. e., after 12 applications of the compound) the first animals showed a distinct epilation. The first (multiply growing) tumours appeared by the 9th week (25 applications). These animals showed no complete epilation (Fig. 2). Warts were present in 50 per cent of the animals by the 12th week (35 applications). In the remaining mice multiple, progressively-growing warts developed in a very short period, so that after about 13 weeks from the beginning of the experiment (40 applications) all the animals had tumours. Ulcerations as well as other symptoms indicating malignancy were seen after 10 weeks (45 applications). Warts appeared simultaneously or at short intervals in the treated (wholly epilated in most animals) skin at varying distances from each other, but they quickly became confluent and formed single tumours (Fig. 3).

The animals endured the treatment with sodium cholate solution well and gained in weight. All the mice survived until they were killed on the 19th week of the experiment (55 applications). The average time of response⁹ in these series was about 12½ weeks.

Acetone as carrier for the carcinogen. — The epilation process

began somewhat earlier than in the foregoing experiment (after about 2 weeks from the beginning of the experiment). But by the 5th week there was a distinct regrowth of the hairs. »The average time of response« in these series was about 6 to 7 weeks (0.3 and 0.03 per cent of the carcinogen).

Dioxane as carrier for the carcinogen. — The first warts developed

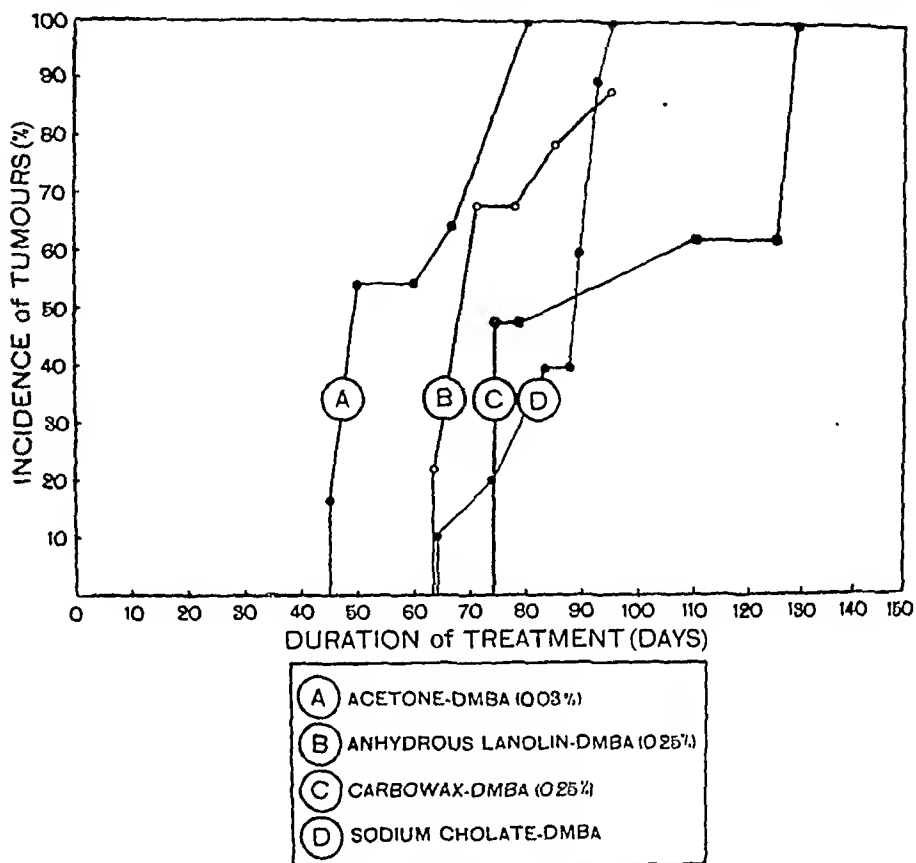


Fig. 1.

Incidence of cutaneous tumours following the treatment with 9,10-dimethyl-1,2-benzanthracene in various solvents.

by the 14th week (40 applications). »The average time of response« was about 14 weeks.

»Carbowax 1500« as carrier for the carcinogen. — The epilation process was (as mentioned earlier⁹) comparatively weak. The first tumours appeared after about 11 weeks from the beginning of the experimental series. »The average time of response« was about 13 weeks.

Anhydrous lanolin as carrier for the carcinogen. — The first tumours appeared by the 9th week of the experiment. »The average time of response« was about 9 weeks.

July 28, 1948.

33 applications (3 x wly) of DMBA
in sodium cholate.



Fig. 2.

Mouse No. 27/0. Multiple warts after 33 applications of the carcinogen in aqueous sodium cholate solution.

Sept. 4, 1948.

51 applications (3 x wly) of DMBA
in sodium cholate.



Fig. 3.

Mouse No. 27/1. Warts becoming ulcerated and confluent. The photograph taken after 51 applications of the carcinogen in aqueous sodium cholate solution.

Microscopic data.

The most common type of cutaneous carcinoma produced by 9,10-dimethyl-1,2-benzanthracene dissolved in sodium cholate solution was the squamous cell carcinoma with cornification (Fig. 4). In the

majority of cases the tumour cells underwent parakeratotic changes instead of normal keratinization.

In some cases the microscopic appearance of the cutaneous changes varied from that mentioned in the foregoing: the hyperplastic and hyperkeratotic epidermis formed a great number of downward projections with invasive properties.

It may be said that the tumours (both innocent and malignant) produced by the carcinogen solubilized in sodium cholate solution differed in no way from those seen in experimental skin carcinogenesis in mice.



Fig. 4.

Squamous cell carcinoma with cornification and parakeratotic changes.
Mouse No. 27/1.

Discussion.

Summarising all the observations mentioned above we may present the following:

(1) By using aqueous solutions of sodium cholate as »solvent« and carrier for 9.10-dimethyl-1.2-benzanthracene it was possible to induce cutaneous tumours in mice.

(2) When used in skin paintings this colloid seemed to be non-toxic to mice. All the animals survived until the end of the experiment.

(3) The tumours induced seemed to have a tendency to grow as multiple tumours from the very beginning.

Association colloids are characterized by the fact that the colloid particles in their solutions are formed by the association of single molecules or ions of the substance in question and that a reversible equilibrium exists between the molecular-disperse and colloidal elements. The state of equilibrium varies with the concentration. At high

dilutions only single molecules or ions are present. Beginning at a certain concentration, which is referred to as »the critical concentration for micelle formation«, association sets in and leads to the formation of colloidal aggregates, micelles. At high concentrations the colloidal substance predominates.

The substances which form association colloids in aqueous solution exhibit as a consequence of their molecular structure a distinct dualism in their behaviour towards water. Their molecules always contain on the one hand a polar group which is hydrophilic in character and forms the water-soluble part of the molecule (*e. g.*, an ionized carboxyl-group). On the other hand, the molecules contain a relatively large hydrocarbon group which is by nature hydrophobic, but also lipophilic, and hence forms the part of the molecule which is insoluble in water, but soluble in fats.

The alkali salts of the higher fatty acids, the soaps, are the earliest known and the most thoroughly studied representatives of the group of association colloids. Investigations of the last decade have attempted to provide information of the structure of the colloid particles, the micelles. At least in the most concentrated solutions they are built up of layers of parallel fatty acid ions with all the ionized carboxyl-groups in each layer pointing in one direction. Two of these layers are always grouped together in a manner such that the hydrophobic hydrocarbon faces of the layers are opposite to each other and the hydrophilic carboxyl faces are outward against the solvent water. The micelles formed in this manner are capable of occluding substances which are fat-soluble but insoluble in water, *e. g.*, hydrocarbons such as benzene, hexane, decane, xylene and the like. X-ray investigations have revealed that the hydrocarbon molecules are situated inside the micelles between the hydrocarbon faces of the layers. Solutions obtained in this manner are both clear and stable. This solubilizing ability is only found in solutions that contain micelles, that is, solutions whose concentrations exceed the critical concentration. As mentioned above, we have found that salts of the higher fatty acids and other association colloids (*e. g.*, sodium myristate, oleate, myristyl sulphate, cholate, »Triton NE«), are also able to solubilize carcinogenic hydrocarbons and we have every reason to assume that their molecules are also contained in the micelles in a similar manner as the lower hydrocarbons.

Some years ago *McBain* and collab.⁶ called attention to the fact that in aqueous solutions the salts of bile acids have properties which in many respects resemble those of the fatty acid salts and other association colloids and should therefore be included in this group. This opinion has since gained considerable favour⁷. Our own studies of the ability of the bile acid salts to bring high- and low-molecular hydrocarbons into solution substantiate this, although it seems that they do in some respects differ from the basic type as exemplified by the ordinary soap^{1, 2, 3}. The critical concentration for micelle for-

mation of the sodium cholate which we have employed in our experiment is about 0.8 per cent (as determined by the fluorescence method)³.

The solubilized hydrocarbon is occluded inside the micelle in a lipophilic environment. The micelles are in turn surrounded by water (or more correctly, by a dilute solution of molecular-disperse soap, etc.). In an association colloid solution we thus have a solution which is a combination of hydrophilic and lipophilic media and which differs in a characteristic manner from most of the ordinary types of solutions, but may in certain respects approximate the conditions extant in the living cell (?).

The solutions under consideration have a low surface tension. They can be easily spread on the skin and penetrate into it readily. In this manner the micelles transport the occluded carcinogen with them. When they come into contact with the fat deposits in the cells they surrender a part of their carcinogen as is shown by our observations with the fluorescence microscope. These show also that the carcinogen enters the living cells. The observations made thus far did not reveal whether this transportation takes place together with the micelles or whether the carcinogen enters the cells through the lipophilic membrane in molecular form.

Our investigations have shown that the carcinogens maintain their activity in association colloid solutions (both 9,10-dimethyl-1,2-benzanthracene and 20-methylcholanthrene, unpublished). Painting with these solutions may offer a milder mode of application than when acetone, benzene, dioxane and other solvents are used. It thus appears that this method of introducing carcinogens solubilized in association colloid solutions would offer a number of advantages over the methods of application previously employed.

Summary.

The evidence presented proves that the epidermis of the mouse skin is capable of producing tumours — both innocent and malignant — when aqueous solutions of sodium cholate are used as carrier and spreader for 9,10-dimethyl-1,2-benzanthracene. Painting with these solutions may offer a milder mode of application than when »ordinary« fat-solvents are used.

The nature and characteristics of association colloids in general are discussed.

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SKIN TUMOURS PRODUCED IN MICE BY CARCINOGENIC HYDROCARBONS SOLUBILIZED IN AQUEOUS SOLUTIONS OF ASSOCIATION COLLOIDS. II.

By *Kai Setälä* and *Per Ekwall*.

(Received for publication February 14th, 1949.)

Clear, homogenous and stable aqueous solutions of carcinogenic hydrocarbons can be prepared with the aid of association colloids, as *Ekwall* and *Setälä* have shown^{1, 2}. These solutions penetrate readily into the tissues and the micelles transport the solubilized carcinogen with them^{2, 3}. Our first task is to investigate whether the carcinogens maintain their tumour producing power in these aqueous solutions. If this is the case, we will compare the carcinogenic potency of the hydrocarbons in the different aqueous associations colloid solutions with their potency in earlier used solvents.

The following will be an account of the results of experiments in which 9,10-dimethyl-1,2-benzanthracene, solubilized in aqueous Triton NE solution, has been painted to the skin of mice.

Technique.

The general investigation technique employed in the present work was essentially the same as that previously used by the writers^{3, 4}.

»*Triton NE*« (Rohm & Haas Company, Philadelphia) is the name of a commercial product of which the colloid forming substance is an alkyl aryl polyether alcohol. In water solutions this material acts as a *non-ionogenic* association colloid forming micelles, which are capable of solubilizing carcinogenic hydrocarbons. The critical concentration for micelle formation of the commercial preparation is about 0.06 per cent.

The Triton NE preparation is a viscous aqueous solution which in

vacuum over phosphorus pentoxide loses 67.6 per cent of its weight, the residue being an extremely viscous liquid. The preparation obviously contains some electrolytic impurities. The p_H value of the twenty

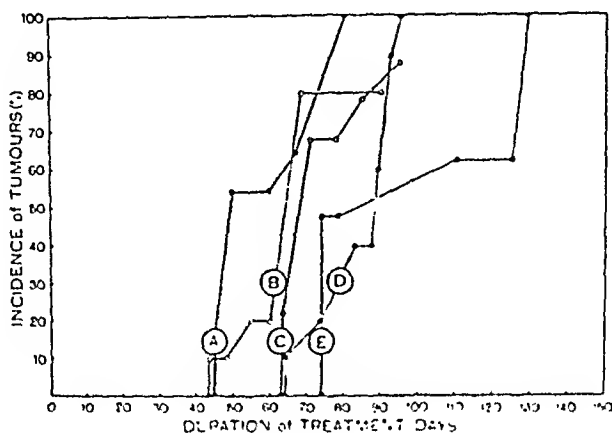


Fig. 4.

Incidence of local cutaneous tumours following treatment with 9,10-dimethyl-1,2-benzanthracene dissolved in:

- A = acetone (0.03 per cent carcinogen),
 - B = »Triton NE« solution (0.07 per cent),
 - C = Anhydrous lanolin (0.25 per cent),
 - D = Sodium cholate solution (0.25 per cent),
 - E = »Carbowax 1500« (solid) (0.25 per cent).
- Abcissae: days (duration of treatment),
Ordinates: per cent (incidence of tumours).

Nov. 11, 1948.

39 applications of DMBA
in Triton NE



Fig. 2.

Cutaneous tumours induced by 39 applications of the carcinogen solubilized in »Triton NE« solution. No distinct epilation of the skin.

Dec. 8, 1948.

50 applications of DMBA
in Triton NE



Fig. 3.

Multiple tumours in a mouse induced by 50 applications of the carcinogen solubilized in »Triton NE« solution. No distinct epilation.

per cent solution is 7.4. The strongly fluorescent »Triton NE« solutions contain about 700 mg. carcinogen in 1000 c.c. of the 20 per cent solution (0.07 per cent).

Results.

In some experimental animals the changes indicating epilation of the skin began by the 3rd week of the experiment. The hair began,



Fig. 4.

Microscopic appearance of the tumour shown in Fig. 2.

however, to regrow. After the 7th week of the experiment all animals were fully haired. There was no distinct epilation in the tumour bearing animals either (Figs. 2 and 3). The first — solitarily growing warts appeared by the 6th to 7th weeks of the experiment (corresponding to 17 applications of the carcinogen). All surviving animals (8) had tumours by the 14th week of the experiment. The average time of response was about 9 weeks (about 28 applications of the carcinogen).

Microscopically the tumours were like those seen in experimental carcinogenesis in general (Fig. 4).

Discussion.

9,10-dimethyl-1,2-benzanthracene maintains its carcinogenic potency in aqueous solutions of the non-ionogenic association colloid Triton NE. The average time of response in different solvents varied very little. In Triton NE solution it was somewhat longer than in acetone but a little shorter than in anhydrous lanolin. »Carbowax 1500« and in aqueous sodium cholate solutions (Fig. 1).

It became evident already in the early work on carcinogenesis that the nature of the solvent plays an important rôle in the carcinogenic response of the tissues. A proper understanding of this »solvent effect«⁷ is still lacking. It has been suggested that this effect might be correlated with differences in the elimination rate of hydrocarbons^{5, 6, 7, 8, 9}. We still know, however, comparatively little about these phenomena. For the investigation of these problems it would be of value if the carcinogenic hydrocarbons could be applied in carriers with different physical and chemical characteristics. In this respect the fairly well characterized solvents which we have examined offer new possibilities. This is the case as concerns the water-soluble polyethylene glycols (»Carbowax«)⁴ as well as the aqueous solutions of the association colloids^{1, 2, 3}. Especially the properties of the latter can be varied within wide limits.

Summary.

Data have been presented that indicate that 9,10-dimethyl-1,2-benzanthracene maintains its carcinogenicity also in »Triton NE« solutions (the colloid forming substance is an alkyl, aryl polyether alcohol).

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THE VASCULAR RESPONSES OF THE MOUSE SKIN TO MUSTARD GAS

By *Leiv Kreyberg* and *Odd Eiwin Hansen*.

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The effects of mustard gas on the living organism have been intensively studied since the introduction of this war gas during the First World War, and the main clinical picture of the local, as well as the general injuries, were well established before the out-break of the last war. The finer mechanism of the development of the tissue damage was, however, little known. In the hope of finding an effective treatment, a large amount of research work was instigated, and the pertinent literature is considerable.

It has been shown that mustard gas reacts with many proteins, and with some of these in a special manner. It has further been established that mustard gas inhibits certain enzymes, hexokinase especially, with ensuing changes in the cell metabolism. *Boursnell* (1948) and *Needham* (1948) have given a valuable review of our present knowledge and its limitations.

Regarding the vascular reactions to mustard gas, the literature gives little information. *Cameron* and *Short* (1944) observed an increased vascular permeability in the skin of rabbits a few minutes after the application of mustard gas, according to a paper quoted by *Cullumbine* (1947). These observations were confirmed by *Cullumbine* and *Rydon* (1946) observing the reactions in rabbits and in man, and by *Young* (1947) in experiments with rats. These students used the intensity of staining with trypan blue as an indication of the vascular reaction. *Young* (1947) also examined the edema quantitatively and found formation of edema in the injured area during the first 12—18 hours after the application of mustard gas. Later the edema disappeared gradually, until the fluid content of the skin again was normal 72 hours after the application.

Cullumbine and *Rydon* (1946), in skin treated with mustard gas, found a leucotaxin-like substance, which again produced an increased vascular permeability (diagnosed by the staining with trypan blue).

They further found that a proteinase (*Beloff and Peters, 1945*) produces this leucotaxin-like substance by digestion of certain proteins, and *Peters (1947)* suggests that a proteinase is of importance for the blister formation.

These observations indicate that vascular reactions are among the first responses of the organism upon the application of mustard gas. The observations by *Ricker (1924)*, *Levy (1929)*, *Kreyberg and Rotnes (1931)*, *Kreyberg (1948)* and others indicate, that it would be of importance to establish whether mustard gas produces true stasis or not and in case, to ascertain the possible role of stasis in the development of mustard gas necrosis.

Pullinger (1947) observed the vascular reactions to mustard gas by direct observations in vivo in the ears of mice. She found that »blood circulates through the lesion for a few days after necrosis has occurred and patent lymphatics traverse it. Vascular occlusion occurs after 6—9 days«. In another passage in the paper is stated that: »The maintenance of a blood circulation after the appearance of coagulation necrosis rules out death by vascular occlusion and infarction. Occlusion occurred 4—7 days later«.

In a series of preliminary experiments the writers of the present paper found a certain lack of agreement with the findings of Pullinger, and we decided to study the problem further, using another technique. The results will be presented below.

Technique.

A strain of hairless mice (recessive hairless) was used in the experiment. The animals were of both sexes, and all of them were adults. Special care was taken that the skin was free from wounds or scars. By means of a capillary pipette a very small drop of undiluted mustard gas was applied in the lumbar region at the centre of the back. In one of the test series mustard gas was also applied to one ear and in the neck. The amount applied always resulted in necrosis, while the general condition of the animals was largely unchanged during the observation period.

The vascular reactions in the areas of application were studied according to the procedure described by *Kreyberg and Rotnes (1931)* and *Kreyberg (1948)*. The colloidal stains, lithium carmine and trypan blue, were injected intraperitoneally at various time-intervals after the application of the gas. The staining of the tissue in the area of application indicated the condition of the vessels and the circulation through them. In addition, intraperitoneal injection of the crystalline in stain, fluorescein, was used, and observed by means of ultra-violet light, as described by *Lange and Boyd (1943)*. Complementary experiments using Indian ink were carried out in parallel series. The ink was injected intravenously in a tail vein, or, when this was impossible, intracardially under ether narcosis. The absence of penetration in the area of application was interpreted as a sign of obstruction of the vessels, if the surrounding vessels contained a profusion of grains of Indian ink.

The observations were confirmed, as the skin was fixed after the experiment, dehydrated, cleared in xylol and embedded in Canada balsam.

In addition, histological examination of the skin of the area of application of the gas was carried out at stages corresponding to the injections of the stains.

Experimental Results.

In the course of the first 5—10 minutes a light hyperemia could be observed followed by a slight cushion-shaped swelling, after which the hyperemia disappeared. In accord with *Cullumbine* and *Rydon* (1946) and *Young* (1947) we found staining in the area of application after few minutes, if lithium carmine was injected immediately following the application of the gas. The animal did not show general staining until 1—2 hours later, and at this time the area of application was intensely red. Lithium carmine is adsorbed to the plasma proteins. The observations may indicate that mustard gas immediately causes an increased permeability of the capillaries. One should note, however, that the intense staining of the injured tissue also may be caused by an increased blood flow, even with a normal permeability. The most intense staining is probably a result of a combination of increased permeability and an increased blood flow. In our experiments the areas of application showed a very intense red colour.

The vascular reactions were studied by injection of the different stains at various intervals of time after the application of the mustard gas.

1. *Lithium carmine.* We injected 0.1 ml of the stain intraperitoneally, and the observations were made when the animal showed general staining. The results are recorded in Table I which shows that lithium carmine produced a strong staining of the tissue in the area of application when the injection was made 1 and 3 hours after the application of the mustard gas. After 6 hours most of the area showed strong red staining, except a small central spot which remained unstained. When the injection was carried out 12 hours or more after the application, the area of application was unstained, but surrounded by an intensely red zone. The area remained unstained for 6 hours after the injection, but became faintly stained in the course of 11 hours.

2. *Lithium carmine and trypan blue.* The experiments of the preceding series were repeated, with the injection of both lithium carmine and trypan blue at successive intervals. Five minutes after the application of mustard gas, 0.05 ml of lithium carmine was injected intraperitoneally. The animal became slightly stained while the area of application showed intense red colour. When 0.05 ml trypan blue was injected later, the blue colour of the rest of the animal contrasted

Table I.
Vital staining with lithium-carmines.

Mouse no	Time between application and injection	Time between injection and observation	General staining of skin	Staining of the treated area
MH 1/VIII/49	1 hour*)	15 minutes	Moderate	Deeply stained.
MH 2/VIII/49	3½ hour	3 hours	Moderate	Deeply stained.
MH 3/IX/49	6 hours	½ hour	None	Most of the area deeply stained, but in the centre a small field is unstained.
		1 hour	Faint	Unchanged.
MH 5/VII/49	13 hours	3 hours	Moderate	Deeply stained halo around the unstained central area.
MH 2/V/49	24 hours*)	2 minutes	Faint	Deeply stained halo around the unstained central area.
		6 hours	Moderate	Unchanged.
		24 hours	Moderate	Deeply stained halo and a faint staining of the centre.
MH 4/IX/49	24 hours	2 hours	Moderate	Deeply stained halo around the unstained centre.
MH 4/V/49	25 hours	5 hours	Moderate	Deeply stained halo around the unstained centre.
		23 hours	Moderate	Faint staining of the centre also.
MH 5/V/49	49 hours	5 hours	Moderate	Deeply stained halo around the unstained centre.
		11 hours	Moderate	Faint staining of the centre also.

*) Intravenous injection.

sharply with the local red colour of the injured area. This observation was made when the animal total was stained, as a rule 1—2 hours after the injection of the trypan blue. The results, given in Table II, agree completely with the results of the experiments with lithium carmine only. The area of application, first red, showed an additional marked blue staining when the trypan blue was injected within 6 hours after the application of mustard gas. The blue staining was,

Table II.

Vital staining with trypan blue (and lithium carmine*).

Mouse no	Time between application and injection	Time between injection and observation	General staining of skin	Blue staining of the treated area
MH 1/IX/49	6 hours	1 hour	None	Deeply stained with small, irregular, faintly stained or unstained fields.
MH 15/IX/49	6 hours	1½ hour	Moderate	Deeply stained with small, irregular faintly stained fields.
MH 7/X/49	12 hours	1½ hour	Moderate	Deeply stained halo around the unstained central area.
MH 14/IX/49	12 hours	1½ hour	Moderate	Deeply stained with a moderately stained central field.
MH 6/VII/49	13 hours	2 hours	Moderate	Deeply stained halo and a faint staining of the centre.
MH 16/IX/49	18 hours	1½ hour	Moderate	Strongly stained halo. Unstained central area with some faintly stained spots along some of the larger vessels.
MH 9/IX/49	18½ hour	2 hours	Moderate	Deeply stained halo around the unstained central area.
MH 10/IX/49	24 hours	1¼ hour	Moderate	Deeply stained halo around the unstained central area.
MH 12/IX/49	24½ hour	1½ hour	Moderate	Strongly stained halo. Unstained central area with some faintly stained spots along some of the larger vessels.
MH 17/IX/49	45 hours	1½ hour	Moderate	Deeply stained halo around the unstained central area.

*) All animals, except mouse no 6/VII/49, were injected with lithium carmine a few minutes after application of mustard gas. The painted area developed a deep red staining. For particulars, see the text.

however, not uniform, showing some irregular light blue spots in the pure red areas 6 hours after the application. Trypan blue injected 12 hours after the application gave in some cases spotted staining, as after 6 hours, while in others there was no blue in the red area, but

own, in which the previous epididymitis had occurred much earlier, however, argue against the general truth of this theory. In view of our knowledge of the extraordinary regeneration capacity of the epithelium it seems hardly probable that an inflammatory defect of the epithelium should persist for such a long time.

On the other hand, it is quite feasible that both in those cases with a previous history of epididymitis and in those without, a fresh inflammation might have arisen with following destruction of the ductular wall and consequential escape of the spermatozoa into the surrounding tissue.

The third possibility, degeneration and atrophy of the tubular epithelium, is favoured by the finding in *Steinberg & Straus's* cases. In my cases there were no general degenerative changes in the epithelium of the epididymis. The focal destruction of the epithelium, discernible in some areas, seems to be quite recent and not due to earlier inflammation.

As mentioned further up, in all of my cases except one, there was a varying degree of tubular dilatation with flattened epithelium and with a stagnation of a likewise varying number of spermatozoa. *Steinberg & Straus's* cases and one of *Orsos's* presented similar pictures. The stagnation and the flattening of the epithelium must presumably be caused by an obliteration of a more distal part of the tubulus due to an active or previous inflammation. That an obliteration of the efferent ductules have this effect on the tubules above the obliteration has been demonstrated partly by studies in the dissecting room and partly by the experimental clamping of the vas deferens or the caudal parts of the epididymis. (*Richter; Naga; Kyrle & Schopper*).

The regular evidence of an increased intratubular pressure, seems to be essential in the arisal mechanism of sperm invasion. It is plausible that the increased pressure may result in a rupture of the distended tubular wall. It is possible — analogous to the rupture of the fornix in pyelovenous reflux — that the pressure alone is sufficient to rupture the wall. It sounds more reasonable, however, to ascribe a possible rupture to the combination of two factors: increased pressure and local decrease in the resistance of the wall due either to an acute inflammation — previous or active — or to a chronic inflammation.

Summary.

In an examination of a relatively large material of non-specific epididymitis sperm invasion of the interstitial tissue of the epididymis was no infrequent by-finding. In some cases the sperm invasion dominated the picture, and the inflammatory changes were mainly localized and most marked around the invading spermatozoa forming granuloma-like foci. Seven cases of this type are described. In addition to the sperm invasion and the localization of the inflamma-

Table III.
Vital staining with fluorescein.

Mouse no	Time between application and injection	Time between injection and observation	General staining of skin	Straining of the treated area
MH 20/IX/49	3 hours	5 minutes	Moderate	Deep, spotted staining.
MH 1/IX/49	6 hours	5 minutes	Faint	Deep, spotted staining.
MH 3/IX/49	6 hours	30 minutes	Strong	Deeply stained.
MH 2/VIII/49	6 hours	5 minutes	Faint	Deep, spotted staining.
		30 minutes	Strong	Deeply stained.
	6¾ hour	5 minutes	Strong	Deeply stained halo around the unstained central area.
		10 minutes	Strong	Faint staining of the centre.
MH 7/IX/49	12¼ hour	5 minutes	Strong	Deeply stained halo. Spotted staining of the central area.
MH 8/IX/49	12¼ hour	5 minutes	Strong	Deeply stained halo. Spotted staining of the central area.
MH 5/VII/49	13 hours	5 minutes	Strong	Deeply stained halo around the unstained central area.
		1 hour	Strong	Deep staining of the centre.
MH 9/IX/49	18½ hour	5 minutes	Strong	Deeply stained halo around the unstained central area.
		1 hour	Strong	Faint staining of the centre.
MH 19/IX/49	21½ hour	5 minutes	Strong	Deeply stained halo around the unstained centre.
MH 4/IX/49	24 hours	5 minutes	Strong	Deeply stained halo around the unstained central area.
		1 hour	Strong	Faint staining of the centre.
MH 10/IX/49	24 hours	5 minutes	Strong	Deeply stained halo around the unstained central area.
		1 hour	Strong	Faint staining of the centre.
MH 17/IX/49	45 hours	5 minutes	Strong	Deeply stained halo around the unstained central area.
		1 hour	Strong	

Fluorescein was usually injected in conjunction with lithium carmine or trypan blue.

Table IV.
Filling of the blood vessels with Indian ink.

Mouse no	Time between application and injection	Amount injected and way of injection	Degree of filling of the blood vessels in general	Degree of filling of the blood vessels in the treated area.
MH 3/VIII/49	3 hours	$\frac{3}{4}$ ml i. v.	Good	Good.
MH 20/IX/49	3 hours	3 ml i. c.	Good	Good.
MH 10/VIII/49	6 hours	1 ml i. c.	Good	Good.
MH 12/VIII/49	12 hours	1 ml i. v.	Good	No filling of the minute surface vessels.
MH 14/VIII/49	15 hours	1 ml i. v.	Not good	No filling of the minute surface vessels.
MH 16/VIII/49	18 hours	i. c.	Good	No filling of the minute vessels. Some of the larger vessels filled.
MH 19/IX/49	21 $\frac{3}{4}$ hour	4 ml i. c.	Good	Some of the smaller and some of the larger vessels filled.
MH*) 3/V/49	25 hours	2 ml i. c.	Good	No filling of the minute vessels. Some of the larger vessels filled.
MH 8/X/49	25 hours	1 $\frac{1}{2}$ ml i. v.	Good	No filling of the minute vessels. Some of the larger vessels filled. Strong injection of the vessels in a halo around the injured area.
MH 9/X/49	26 hours	1 ml i. v.	Good	»
MH*) 7/V/49	60 hours	1 ml i. c.	Good	»
MH*) 8/V/49	74 hours	$\frac{1}{2}$ ml i. v.	Poor	No filling of the minute vessels. Some of the larger vessels filled.
MH*) 9/V/49	8 hours	$\frac{1}{2}$ ml i. v.	Good	No vessels filled in the injured area, but the area is surrounded by a halo of engorged vessels.

All animals, except those marked *), were injected with lithium carmine a few minutes after application of mustard gas. The painted area developed a deep, red staining.

vessels filled with Indian ink, even more than in the surrounding tissue, could be observed during the first 48 hours. After the following 24 hour period a few of the smaller vessels only were filled with Indian ink. After 4 days none of the smaller vessels were filled, while the larger ones still contained Indian ink. At the same time was observed, that the injured ear generally contained more Indian ink than the other.

When mustard gas was applied simultaneously to the ear and the neck, and trypan blue was injected 24 hours later, one found that the area of application in the neck remained unstained, while the entire area of application on the ear took on an intense blue colour. On injection of Indian ink 24 hours after the application of the gas, the vessels of the ear became filled with Indian ink, while the area of application in the neck showed complete absence of Indian ink.

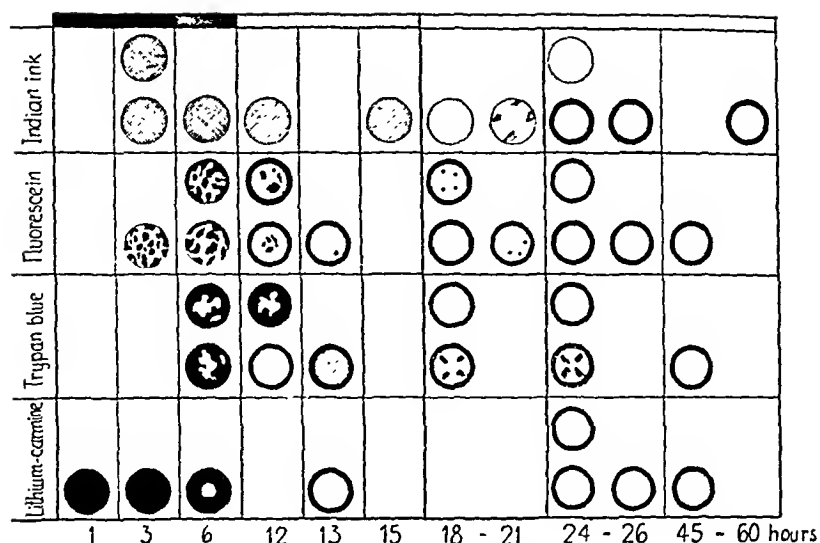
Microscopic investigation of skin injured by mustard gas, taken 1 and 3 hours after the application, showed hyperemia and some infiltration of leucocytes. In injured skin taken 6, 12 and 24 hours after application an increasing degeneration of the cells was found, with pyknotic nuclei and nuclei showing karyorrhexis. After 24 hours there was in addition a considerable haemorrhage. In addition to these degenerative changes, the skin taken 48 hours after the application also showed manifest necrosis in large areas of the section.

Discussion and Conclusions.

The present paper confirms the previously known findings, that vascular changes in the skin constitute an immediate reaction to mustard gas. The increased staining of the tissue in the area of application of the gas, which occurs within a few minutes after the application, is probably caused partly by an increased circulation in, and partly by an increased permeability of, the minute vessels.

In the course of a few hours the early reaction is followed by a different one, as shown by the decreasing staining of the area of application, after injection of lithium carmine, trypan blue or fluorescein. An increasing number of the smaller vessels become occluded and finally the blood flow stops in the entire area. The occlusion was confirmed by the intravascular injection of Indian ink, the ink failing to enter the small vessels.

The staining showed that the circulation was retarded within 6 hours, possibly even earlier. After 12 hours there seemed to be an almost complete cessation of the blood flow in the superficial capillary network of the area of application, the vessels being largely devoid of stain or Indian ink. An examination with binocular microscope of



This diagram summarizes the observations of the vascular response in the different intervals after application of mustard gas. The completeness of filling of the vessels by Indian ink and the degree and the completeness of staining is indicated by the figures. Fluorescein is the stain that gives the first indication of a decreased staining, after the initial very strong colouring. Trypan blue and lithium carmine give a less graded response, and the filling of the vessels by Indian ink is the least sensitive test. The first indication of a reduction of the staining is found 3 hours after the application of mustard gas. The reduction is definite in the course of 6 hours. The circulation is brought to a complete stand-still after some 15—18 hours. The heavy »ring« indicates the development of a hyperemic zone of demarcation.

whole skin preparations from skin injected with Indian ink showed, that while the small superficial vessels generally were occluded, the blood still flowed through the arterioles and veins of the deeper layers as well as in the capillary network of the skin muscle. The larger vessels of the area of application did not all become occluded until 18—24 hours after the application. In some of the larger vessels there was still at this point some blood flow, and in a few cases they even seemed to be permeable for the stains. When the circulation had stopped in the injured area, one found the field surrounded by a hyperemic halo.

Even the initially unstained areas were gradually totally stained, by lithium carmine after a few hours, by fluorescein within one hour. This is probably due to a diffusion of the stain from the surrounding tissue, since the Indian ink showed occlusion of the vessels. There was no indication that the disturbance of the circulation was of a passing, functional nature.

Also in the ears the small vessels in the area of application were occluded, but only after a longer period than in the skin of the body.

viz. 3—4 days after the application. We have no explanation of this difference.

We have thus, in the skin of the body, found an earlier occlusion of the vessels than that described by Pullinger. On the other hand, we have confirmed the somewhat slower reaction in the ears, even though we believe that most of the vessels are occluded sooner than maintained by Pullinger. This applies to the smaller vessels. The larger vessels of the ear permit blood flow for a considerably longer period. This shows that regional factors must be taken into consideration in a discussion of the vascular responses.

The circulatory disturbances indicate the occurrence of stasis, but it has been impossible to confirm this by direct observation of the vessels *in vivo*.

The stasis is usually irreversible and leads to necrosis. To what extent this vascular reaction is responsible for the subsequent necrosis in connection with local mustard gas injuries has not yet been ascertained.

Undoubtedly mustard gas kills the cells directly when the doses are sufficient. *Fell* and *Allsop* (1948) found that liquid mustard gas, or a saturated vapour of the gas in cultures of chicken embryo rapidly kills the cells by fixing or coagulation. Low saturation of the mustard gas vapour kills the cells slowly. *Berenblum* and *Schoental* (1947) found that mustard gas and nucleoproteins *in vitro* form an insoluble precipitate, an observation which may well be the explanation of the directly destructive effect of the gas on cells. The increasing degeneration of the cells which was found developing parallel to the circulatory disturbances may indicate that the doses used in the present experiments were sufficient to kill the cells, without the additional effect of the vascular responses. The manifest necrosis which was found in a section taken 48 hours after the application of the gas gives no conclusive evidence.

Our observations have not established whether the local necrosis is a direct destruction of the cells, or a result of stasis. But we cannot, on the other hand, accept Pullinger's statement that the »maintenance of the blood circulation after the appearance of coagulation necrosis rules out death by vascular occlusion and infarction,« on the basis of our present knowledge. Further experiments are needed in order to solve this problem.

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DETERMINATION OF BACTERIAL RESISTANCE TO PENICILLIN, SULFATHIAZOLE AND STREPTOMYCIN

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The methods employed for measuring of the potency of antibiotics may also be used for determination of the resistance of bacteria to these antibiotics. The choice of method (cup, cylinder, diluting, etc.) will depend on its purpose. For routine examination of a fairly large number of specimens it seems preferable to use a simple and rapid method, here we have chosen the disc method given by Vincent & Vincent (1944).

The disc method.

From all the specimens a direct culture is made on a »resistance plate«, i. e., a 10 % blood agar plate, 6–8 mm. thick, 9–10 cm. in diameter. 3 sterile »discs«, i. e., circular pieces of filter paper, 15 mm. in diameter, are placed at the same mutual distance on the inoculated plate. On each disc is placed respectively one drop of

Penicillin	200 I. U. per cc.
Sulfathiazole	0.5 % aqueous solution
Streptomycin	10,000 I. U. per cc.

The plates are incubated, and on the following day the diameters of the inhibition zones in mm. are read. The strains are divided after their degree of resistance into 4 groups, recorded in the same way for the three solutions:

0	≤ 15 mm.	≡ resistant.
+	16–24 »	≡ slightly sensitive, relatively resistant.
++	25–34 »	≡ moderately sensitive.
+++	≥ 35 »	≡ strongly sensitive.

Relatively resistant strains are not affected by ordinary clinical dosage, but possibly by larger doses. When a specimen yields a bacterial strain in pure culture, its resistance can be read directly from the resistance plate. But when a specimen gives a mixed culture, it usually will be necessary to

isolate the individual strains and repeat the resistance determination on the pure cultures.

Besides on the resistance plate, all the specimens in the following were examined also by inoculation on 5 % blood agar in such a way that single colonies could be isolated easily. Further, from all urines and other specimens in which Gram-negative rods were mostly to be expected, cultures were made on «blue plates» (Conradi-Drigalski medium). Specimens that possibly might contain Pfeiffer bacteria (pus from the ear, sputum and spinal fluid) were examined for resistance not only on blood agar but also on *Levinthal plate* (see below). The resistance of meningococci was determined on ascites agar plate incubated in carbondioxide atmosphere.

Finally, from all the specimens cultures were made in *serum broth* (broth with 5 % ox serum and 1 % glucose). If the serum broth cultures showed growth within 48 hours subcultures were made on 5 % blood agar. If growth was obtained only in serum broth, not on the corresponding plates, an attempt was made to determine the resistance on the serum broth culture. It has to be kept in mind, however, that with such determinations contamination is very likely to occur.

Specimens of sputum, spinal fluid and pleural effusion were injected intraperitoneally in a dose of 0.5 cc. into mice in order to disclose the possible presence of pneumococci, not revealed by the direct examination. The specimens of urine, spinal fluid and pleural effusion were centrifuged prior to the cultivation. From the sediment a smear was made, stained after Gram and appraised with regard to the character and amount of bacteria present. Gram-stained smears were made also from specimens of pus.

The penicillin and streptomycin solutions used for the resistance determinations were made from commercial preparations (e. g., «Penicillin Leo» and «Streptomycin Pfizer»). The contents of the ampullas were dissolved in saline with pH 7.38. It is to be kept in mind that streptomycin increases in volume on being dissolved. The solutions have to be kept in alkali-free glass containers. The penicillin solutions ought to be renewed at least once a week whereas the streptomycin solution is considerably more stable. The sulfathiazole solution here employed is a 0.5 % aqueous solution of «Chemosept» (Ferrosan). A few drops of sodium hydroxide (n/5 NaOH) are added in order to promote the solution. The potency of the three fluids is controlled not infrequently by resistance determination on the standard strain staphylococcus 209 P.

Investigation into the employed method.

The procedure here employed for resistance determination has been tried out thoroughly in various ways. Experiments have shown that discs with diameters of 10, 15 and 20 mm. under otherwise the same experimental conditions give inhibitory zones of the same size. With the employment of large discs these may be removed soon after the fluid has been dropped on them. The same inhibitory zone was obtained by removal of the disc after 1, 2, 5, 10 or 15 min. after application of the fluid as by leaving the disc alone till the following day. The same result is obtained whether the plate is inoculated before the disc is put on, or whether the disc is placed on the noninoculated plate, moistened and removed and then the plate is inoculated (Jensen & Kiær, 1948).

With the diluting method the size of the inoculum is of great significance to the determination of the resistance to sulfathiazole and streptomycin. On determination of bacterial resistance to penicillin and streptomycin after the disc method the density of the culture plays a minor role. It is the general rule, however, that the fewer bacteria in the inoculum the larger are the inhibition zones or, in other words, the greater is read the bacterial sensitivity.

In the sulfathiazole disc method the character of the inoculum plays a decisive role in certain cases (see below).

As mentioned before, the resistance plate here employed is a 10 % blood agar plate, 6—8 mm. thick. When thinner plates are used the inhibition zones will be larger, but when the plate is of more than a certain thickness (6—8 mm.) its exact thickness is of minor importance. The blood plates contain 10 % defibrinated horse blood in beef agar, i. e., 1.8 % agar in beef broth with 1 % peptone («Orthana special»), 0.3 % NaCl and 0.2 % $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$.

For determination of the resistance of Pfeiffer bacilli *Levinthal agar* has been used. With a view to the sulfathiazole determinations, 10 % blood is used in this medium, too. Removal of the red blood cells or addition of 0.5 % glucose and 10 % yeast extract give no better results. The Levinthal plates are prepared just as the above mentioned 10 % blood plates — only with this difference, that the mixture of blood and agar is placed on boiling water bath for 5 min. before the plates are poured. The inhibition zones for the same bacterium are the same on a Levinthal plate and a 10 % blood agar plate.

A slight difference in the concentration of the solutions employed is of no importance with this method. Thus, for instance, after the disc method the standard staphylococcus 209 P shows the following inhibition zones with streptomycin solutions of different concentrations:

Staph. 209 P	10.000	2000	1000	500 U./cc. streptomycin
	29	27	25	23 mm.

Determination of resistance to penicillin.

A comparison has been made between resistance determinations after the disc method and after the diluting method. This gives a good impression of which growth-inhibiting concentrations of the respective solutions correspond to the different inhibition zones (Table 1).

Table 1.

Comparison between the disc method and the diluting method for determination of resistance to penicillin, sulfathiazole and streptomycin.

Degree of resistance	Disc method: Zone of inhibition in mm. for pen., su. and str*)	Diluting method: Inhibition of growth for:		
		Pen. in units per cc.	Sulfathiazole conc.**)	Streptomycin in units per cc.
Resistant 0	0—15	> 5	> 1:10 000	> 100
Relatively resistant +	16—24	5 - 0,5	1:10 000—1:20 000	100—10
Moderately sensitive ++	25—34	0,5—0,1	1:40 000—1:80 000	10—2
Strongly sensitive +++	≧ 35	< 0,1	< 1:80 000	< 2

*) pen. = 1 drop 200 units per cc. of penicillin.

su. = 1 » 0.5 % sulfathiazole.

str. = 1 » 10.000 units per cc. of streptomycin.

**) The given concentrations apply to pneumococci.

The *penicillin resistance* of about 100 strains was examined both after the disc method and after the diluting method in serum broth and in blood agar (when kept in ice-box, blood agar with addition of penicillin keeps serviceable for more than one week). The three methods were found to agree very well. On the basis of the results from these experiments the values recorded in Table 1 were set up.

As the penicillin concentration obtained in the blood of the patients under ordinary dosage (about 300.000 units daily) only for a short time exceeds 0.5 U./cc., we have to expect that the strains found by resistance determination to be relatively resistant — *i. e.*, inhibited of 0.5—5 U./cc. — are not yielding to an ordinary dosage of penicillin.

Determination of resistance to sulfa preparations.

Determination of resistance to *sulfa preparations* is associated with certain difficulties, as both the culture media here employed and the bacterial cultures contain substances inhibitory to the effect of the sulfa preparations. Harper & Cawston (1945) state that the sulfa-inhibiting substances may be neutralized by a factor in the red blood cells from horse. These authors found that hemolyzed blood (5 %) is more effective in this respect than in nonhemolyzed blood.

In a number of resistance experiments, after the disc method as well as after the diluting method in plates, I found no difference from the employment of hemolyzed and nonhemolyzed horse blood. Both the bacterial growth and the inhibition zones gave uniform findings. On the other hand, more pronounced results were obtained with 10 % blood than with 5 %. Further, the peptone content of the plate may also be of significance to the sulfa resistance determination, but when a sufficient amount of horse blood is employed (10 %), the peptone effect does not assert itself.

The other difficulty in sulfa resistance determinations is the amount of sulfa-inhibiting substances in the bacterial cultures. This fact may in part be counteracted by employment of a diluted culture for inoculation of the resistance plates. If by direct resistance determination on the material or on a pure culture a streptococcus is found to be resistant to sulfathiazole, the examination is repeated with a culture diluted 1:200, and in many cases the strain will now prove sensitive to sulfathiazole.

Some bacteria show an inhibition zone for sulfathiazole that is irregular in outline, with streaks of colonies in the zone — *i. e.*, a so-called »partial inhibition«. When the examination is repeated with diluted culture, as a rule, a clear, well-defined inhibition zone will be found.

In the present material no bacteria other than streptococci have proved sulfa-resistant in undiluted culture, while in the corresponding

diluted culture they were found to be sensitive. All other bacteria which in undiluted culture were found to be sulfa-resistant have also proved resistant on their examination in diluted culture.

Table 2.

Sulfathiazole. — Resistance determination on different strains in undiluted and diluted cultures.

The figures give the diameters of the inhibition zones in mm. on employment of the disc method on 10 % blood agar. To each disc is added 1 drop of 0.5 % sulfathiazole.

Strain:	No.	Culture dilution		
		Undiluted	1:100	1:1000
Staph. aureus	1106	0	0	0
— —	1562	35 partly	35	35
— albus	1059	0	0	0
Strept. hemol.	919	0	45 partly	50
— —	612	0	34 partly	36
— —	729	30 partly	36	36
Enterococcus	997	0	0	0
Colibacterium	1087	18 partly	23	24
Proteus	1003	0	0	0
Pyocyanus	1032	0	0	0
—	1027	25 partly	28 partly	30
Gram-neg. rod	1025	28	28	29

For determination of sulfa resistance in the present material sulfathiazole was employed in the form of »Chemosept« (Ferrosan). For the sake of comparison, also the effect of other sulfa preparations has been examined, namely: sulfonamide, sulfapyridine (»M. & B. 693«), sulfadiazine and phthalylsulfathiazole (Pharmacia Ltd.) all in an 0.5 % aqueous solution.

All the strains which after the disc method are found to be resistant to sulfathiazole prove also resistant to the other four sulfa preparations mentioned, employed in the same concentration. On examination of 20 strains (diluted 1:1000), of different bacterial species and differing in sensitivity, sulfathiazole and sulfonamide are found to give the largest inhibition zones, and sulfapyridine is found to be almost just as active, whereas sulfadiazine and phthalylsulfathiazole show smaller inhibition zones. Whether the outcome of these experiments in vitro corresponds to the clinical effect of the respective substances is a question about which nothing may be said here. Various aspects of the organism — *e. g.*, capacity for absorption and excretion of the substances — will play a role.

Comparison between the disc method and the diluting method in fluid media is difficult to carry through in the case of a sulfa prepara-

tion. In the first place, sulfa preparations are bacteriostatic, not bactericidal. The character of the medium, the size of the inoculum, the rate of growth of the bacteria, the point of time for the reading of the result, and other factors will have influence on the outcome. Furthermore, as mentioned before, sulfa-inhibiting substances are formed during the growth of the bacteria, and this circumstance plays an even greater rôle in resistance determination performed in fluid medium than after the disc method on blood agar.

Only the bacteria that form a very slight amount of sulfa-inhibiting substances or none at all may show a fairly good agreement between resistance determinations on blood agar after the disc method and in serum broth after the diluting method. The findings for pneumococci in this respect are recorded in Table 1.

Under ordinary clinical treatment with a sulfa preparation (about 6 g. daily) the concentration of this remedy in the blood will be about 5—10 mg. % ($= 1:20,000 - 1:10,000$). Thus it should be possible with ordinary dosage successfully to combat strains of moderate or marked sensitivity. The values in Table 1 apply to pneumococci and other bacteria showing agreement after the disc and diluting methods. Strains that are found sulfa-resistant after the disc method have always proved resistant after the diluting method, too.

Determination of resistance to streptomycin.

Determinations of the resistance to *streptomycin* are found on the whole to show concordance between the disc and the diluting method (Table 1). Strains which after the disc method are resistant are inhibited after the diluting method by about 100 U./cc. or more; and the strains which after the disc method are found to be relatively resistant are inhibited in fluid media by about 10—100 U./cc. streptomycin.

Patients who have been treated with an ordinary dose of streptomycin (1—2 g. daily) show in their blood a concentration of 10—20 U./cc. Strains which after the disc method are found to be moderately or highly sensitive to streptomycin are inhibited in diluting tests by less than 10 U./cc. and should thus be accessible to ordinary dosage. Strains that are found to be relatively resistant will in practice prove resistant to ordinary dosage. What was said about sulfa preparations applies to streptomycin, too: it is bacteriostatic, not bactericidal. Hence determination of the resistance of bacterial strains in fluid media meets with similar difficulties as mentioned for sulfathiazole. Under constant experimental conditions, however, the diluting method may very well be employed for determination of bacterial resistance to streptomycin.

The media here employed (blood plates and serum broth) have pH 7.3. As pH 8 is recommended as most suitable for streptomycin experiments, comparative tests were made with media having pH 7.3

and pH 8, respectively. They showed no difference in the outcome of the experiments performed.

According to the definition one streptomycin unit is 50 times smaller than one penicillin unit. One unit of streptomycin is the amount inhibiting the growth of the standard staphylococcus in 1 cc. culture, while one unit of penicillin is defined as the amount inhibiting the growth of the same strain in 50 cc. culture. As I had been working with penicillin for some time before commencing the experiments with streptomycin I chose the streptomycin dose for the disc method in proportion to the penicillin concentration already employed, namely: 10.000 U./cc. and 200 U./cc., respectively.

In comparative tests after the disc and the diluting methods, however, the proportion between the concentrations of penicillin and streptomycin giving inhibition of growth after the diluting method is not 1:50 but 1:20. While I am unable to explain this observation, it is still to be mentioned that the comparison here carried out may be accepted but very approximately. Besides, it is to be kept in mind that in the definition regard is paid only to the effect of the remedies on one strain, whereas in the comparative tests we are dealing with an average effect on different bacterial species.

The present material. Diagnose.

The bacteria found in the present material are divided into four groups after their behavior to the Gram staining. Within the *Gram-positive cocci* the following diagnoses are made: staphylococcus aureus, staph. albus, hemolytic streptococcus, nonhemolytic streptococcus, enterococcus, enterococcus-like streptococcus, pneumococcus, and Gram-positive cocci not further defined (Table 3); altogether 3457 strains of Gram-positive cocci have been tested.

All the enterococcus strains examined ferment sorbitol and are sulfa-resistant. The latter property is so constant that it may be employed diagnostically. The »enterococcus-like streptococci« are strains resembling true enterococci as to their morphology and resistance, whereas they do not ferment sorbitol.

The other large bacterial group, the *Gram-negative rods*, are divided into the following groups: colon bacilli (*Escherichia*), coliform rods, *Klebsiella* bacteria, proteus, pyocyaneus, alkaligenes, *Salmonella* bacteria, Pfeiffer bacteria, and the collective group of »Gram-negative rods not further defined«. Altogether 2503 strains of Gram-negative rods have been examined.

»Colon bacilli« designates Gram-negative rods that form indole and ferment mannitol (with gas production), lactose, often saccharose, and which grow in ammonium glucose but not in ammonium citrate. Strains deviating from this definition (*e. g.*, strains that form no indole, or do not ferment lactose, or are able to grow in ammonium ci-

trate) are here summarily designated as »coliform rods«, although I am quite aware that such strains anyhow may belong to »*Escherichia coli*«. As to the differential diagnosis between colon and *Klebsiella* strains, the reader is referred to a recent paper by Käuffmann (1949).

In the State Serum Institute, during one year (from Dec. 1, 1947 to Nov. 30, 1948) altogether 5998 strains, derived from 4781 specimens have been resistance-tested.

Table 3.

Resistance of the strains to penicillin, sulfathiazole and streptomycin.
The figures (except the numbers of strains) give the percental distribution.

[illegible]

Determination of resistance.

Table 3 gives the resistance observed for all the strains. Summarily it may be said that the Gram-positive cocci (with exception of enterococci and enterococcus-like streptococci) on the whole are sensitive to penicillin, whereas the Gram-negative rods (with exception of some Pfeiffer bacteria) are resistant to penicillin. Most of the Gram-positive cocci (excluding enterococci and enterococcus-like streptococci) are sensitive to sulfa preparations, whereas most of the Gram-negative rods are found to be resistant to these remedies. Leaving out the Pfeiffer bacteria and colon bacilli, 68 % of the Gram-negative rods are found to be sulfa-resistant. Practically all Gram-positive cocci (with exception of enterococci and enterococcus-like streptococci) and more than 2/3 of the Gram-negative rods are found to be sensitive to streptomycin. The numbers of strains of Gram-negative cocci and Gram-positive rods are too small to allow of any generalizing estimation of their resistance.

Gonococci and tubercle bacilli are examined in special departments. Cultivation with regard to the possibility of anaërobic bacteria is performed only on special request.

Occurrence and resistance of the different bacteria.

We will now review the individual bacterial species in the sequence in which they are entered in Table 4.

Staphylococcus aureus. A majority of the 624 strains examined were demonstrated in pus from the ear (356 strains = 57 %) and abscesses. In the urine staphylococcus aureus was found in pure culture but exceptionally. Thus it appears as if urinary infection with staph. aureus is rare, at any rate in adults. In the present material no urine from children has been examined.

In most cases, staph. aureus is highly sensitive to penicillin, from moderately to highly sensitive to both sulfathiazole and streptomycin. It is to be mentioned that at the border of the penicillin inhibition zone the staphylococci may undergo some change in appearance and resemble pneumococci. Altogether 8 % penicillin-resistant strains of staph. aureus have been demonstrated. Sulfa-resistant strains are somewhat more numerous (19 %). In contrast hereto, no streptomycin-resistant strain of staph. aureus has been demonstrated.

Staph. albus. Altogether 612 strains were examined mostly from otitic pus and from urines. On the whole, staph. albus may be looked upon as a saprophyte or contaminating microorganism. Most of the strains are sensitive to penicillin, sulfathiazole and streptomycin.

Hemolytic streptococci are represented in this material by 445 strains, chiefly from otitic pus (53 %) and from throat swabs (25 %). A majority of these throat swabs come from patients with scarlet fever.

Table
Distribution of the bacteria in the present material.

Gram	Bacterium:	Total No. of strains	Ear	Nose	Maxillary and frontal sinuses	Eye	Sputum	Throat	Pleural effusion	Spinal fluid	Blood
Gram-positive cocci	Staph. aureus	624	356	7	1	2	28	9	3	2	4
	— albus	612	275	6	3	1	56	13	—	5	6
	Streptococ, hemolyt.	445	236	11	—	—	30	112	—	3	2
	— non-hemolyt.	582	104	9	3	—	220	56	1	3	31
	Enterococcus	633	42	3	—	—	11	7	1	1	6
	Enterococcus-like strept.	84	11	—	—	—	7	—	—	—	—
	Pneumococcus	259	143	7	7	1	65	6	3	15	—
	Gram-pos. coc., not further defined	218	71	2	—	—	36	5	—	—	1
Gram-negative rods	Colibact.	493	10	—	—	—	21	4	2	2	2
	Coli-like rods	552	17	1	—	—	52	8	—	4	—
	Klebsiella	206	—	—	—	—	5	—	—	—	—
	Proteus	382	31	1	—	—	6	2	1	1	—
	Pyocyaneus	173	69	—	—	—	2	1	—	—	—
	Alkaligenes	20	6	—	—	—	1	—	1	—	—
	Salmonella	7	1	—	—	—	—	—	—	—	1
	Pfeiffer	59	13	—	2	—	6	—	—	37	—
	Gram-neg. rods, not further defined	611	86	1	2	—	64	5	1	3	—
Gram-neg. cocci	Meningococcus	6	—	—	—	—	—	—	—	6	—
	Gram-neg. coc., not further defined	6	—	—	—	—	5	1	—	—	—
Gram-pos. rods	Subtilis	5	4	—	—	—	—	—	—	—	—
	Gram-pos. rods, not further defined	20	13	—	—	—	2	—	—	—	—
	Monilia albicans (fungus)	1	—	—	—	—	—	—	—	—	—
	Total	5998	1488	48	18	4	617	229	13	82	53

The hemolytic streptococci are all sensitive to penicillin and streptomycin, even though some of the strains (14 %) only are slightly sensitive to streptomycin. As to sulfathiazole, a great majority of the strains (72 %) are highly sensitive to this remedy. 17 % are found resistant to sulfathiazole when undiluted cultures are employed, but

4.
The figures give the number of strains.

Urine	Peritoneum	Cervix	Prostate	Urethra	Bile	Pus from intestine	Feces	Pus, abscess	Fistular pus	Osteomyelitis	Arthritis	Wounds	Cultures of unknown origin
59	—	8	—	1	—	—	—	81	23	19	5	7	9
153	4	39	7	3	—	—	—	27	6	2	—	2	4
1	1	—	—	—	—	—	—	13	3	1	—	3	29
70	1	19	6	2	—	2	1	16	5	2	—	4	27
472	4	23	1	3	3	—	3	24	13	5	1	6	4
50	1	6	1	—	—	1	—	2	1	2	—	—	2
—	1	—	—	—	—	—	—	2	—	—	1	1	7
48	—	4	4	1	—	—	—	19	13	1	1	6	6
367	21	20	2	2	4	7	2	10	10	—	1	5	1
437	8	1	2	—	3	2	2	4	7	1	—	2	1
198	—	—	1	—	—	—	—	—	—	—	—	1	1
314	3	—	—	2	1	2	1	10	4	—	—	2	1
83	3	—	1	1	—	—	—	1	5	1	—	5	1
7	—	—	—	—	—	—	—	—	2	—	—	—	3
—	—	—	—	—	—	—	1	—	—	—	—	—	4
—	—	—	—	—	—	—	—	—	—	—	—	—	1
403	—	5	2	1	2	1	—	11	4	3	—	6	1
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	1	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
2666	47	125	27	16	13	15	10	221	106	37	9	51	103

with cultures diluted 1:200 only 4 % sulfa-resistant strains are seen.

On 300 strains of hemolytic streptococci an attempt was made to group and type the individual strains.*) 144 strains are designated as

*) I am greatly obliged to Dr. Knud Skadhauge, the State Serum Institute, for his typing of the hemolytic and non-hemolytic streptococci.

Ax, i. e., they belong to Lancefield's group A but cannot be type-determined; the other strains are typed as well as grouped. One strain (from otitic pus) belonged to group C (C21), all the rest to group A, being distributed on types 1, 4, 22 and 25. Type 4 is the most frequent (61 strains), then follow types 1, 22 and 25 with respectively 45, 40 and 9 strains.

Hemolytic streptococci isolated from the throat are easier to type than are strains from otitic pus. Of the throat strains 87 % could be typed as against 40 % of the ear strains. From the other specimens an average of 42 % was typed.

Non-hemolytic streptococci. Altogether 582 strains of this category were examined. They originated chiefly from sputa, otitic pus and throat swabs. Most of the strains of non-hemolytic streptococci are sensitive to penicillin, sulfathiazole and streptomycin, but on comparison to the hemolytic streptococci we here meet with more strains that are resistant to penicillin and sulfathiazole.

What has been said of the hemolytic streptococci applies also to the non-hemolytic: that it can be decided whether or not a given strain be sulfa-resistant only when the determination is performed on diluted culture. Of 49 strains of non-hemolytic streptococci which in undiluted culture are sulfa-resistant, 22 — i. e., nearly one half — are found to be sensitive in diluted culture.

On several of the strains, type determination was performed by means of fermentation tests. A great majority of these strains are strept. mitis, and only a few are strept. equinus and strept. bovis.

Enterococci. Altogether 633 strains were found, isolated chiefly from urine (75 %), but also not infrequently from otitic pus, cervical swabs, and pus from abscesses and fistulas. Enterococci are but slightly sensitive to penicillin. 57 % of the strains were found to be penicillin-resistant, 33 % relatively resistant, 10 % moderately sensitive, and none strongly sensitive. As mentioned, all the strains were found to be resistant to sulfathiazole, also on examination in diluted culture. Enterococci are somewhat more sensitive to streptomycin than to penicillin.

«*Enterococcus-like streptococci*». Altogether 84 strains of this category are recorded. The occurrence of this group of bacteria corresponds fairly well to that of the enterococci. Thus a majority of them (60 %) were isolated from urine. The enterococcus-like streptococci are somewhat more sensitive to penicillin and to streptomycin than are enterococci (Table 3). All the strains are resistant to sulfathiazole, also in diluted culture.

Pneumococci. The material contains 259 strains of pneumococci, obtained chiefly from otitic pus and sputa. In otitic pus, staphylococci

and hemolytic streptococci are the bacteria most frequently found; then follow pneumococci (143 strains from 136 patients). The most frequent types here are: 19, 1, 3, 6 and 14. This type distribution corresponds fairly well to the distribution found in a larger material (Mørch, 1949).

In sputa, next to non-hemolytic streptococci pneumococci are the bacteria most frequently demonstrated. In 65 sputa the most frequent types are: 15, 3, 19, 1 and 20. The frequent occurrence of type 15 is a striking observation compared to the type distribution in a larger material in which types 1, 2 and 3 dominate in the sputa (Mørch, 1949).

The resistance of the pneumococci has a great resemblance to that of the hemolytic streptococci. All the pneumococcus strains are sensitive to penicillin and sulfathiazole. All the strains are also sensitive to streptomycin, a few of them (7 %), however, only in a slight degree. There is no demonstrable difference between the resistance of the individual types.

Several strains (218 altogether) have not been referable to the mentioned group of Gram-positive cocci and are therefore here collected under the term »*Gram-positive cocci not further defined*«. Some of these strains resemble staphylococci but on account of their reactions in mannitol and citrate plasma they could not be referred to the groups aureus or albus. Most of these strains are sensitive to penicillin, sulfathiazole and streptomycin but a not inconsiderable part of them are resistant (see Table 3).

Among the *Gram-negative rods* the colon group is represented most abundantly. Altogether 493 strains of colon bacilli are examined, and 552 strains of coliform bacteria. Strains of the *colon group* are found quite predominantly in urine (74 % of colon bacilli and 79 % of the coliform rods).

Coli bacteria and coliform rods show the same behavior to penicillin, 99 % of the strains in both groups being resistant, and only 1 % being relatively resistant. Only 1/3 of the coli bacteria (32 %) are sulfa-resistant, whereas about 3/4 (72 %) of the coliform rods are sulfa-resistant. Likewise, coli bacteria are more sensitive to streptomycin than are the coliform rods. In the colon group the resistance to streptomycin is in 90 % of the strains associated with resistance to sulfathiazole.

Klebsiella. 206 strains of the *Klebsiella* bacteria are isolated almost exclusively from specimens of urine (96 % of the strains). 5 strains (2 %) are demonstrated in sputa. These Gram-negative capsulated bacteria are the most resistant of the Gram-negative rods. All the examined strains are penicillin-resistant, and most of them are also resistant to sulfathiazole (89 %). Further, a little over 1/3 of the strains (36 %) are resistant to streptomycin.

Altogether 382 strains of *proteus* are examined, found chiefly in

specimens of urine (82 %), though not infrequently also in otitic pus (8 %). 89 % of the proteus strains are penicillin-resistant, and 10 % are slightly, 1 % (3 strains) moderately, sensitive to this antibiotic. Most of the strains (73 %) are resistant to sulfathiazole. 85 % of the proteus strains are sensitive to streptomycin.

By means of fermentation tests, 40 proteus strains are divided into 3 types that are found uniform in their resistance to sulfathiazole and streptomycin, whereas they appear to differ in their sensitivity to penicillin. Among these 40 strains 8 are slightly sensitive to penicillin, while the remaining are penicillin-resistant. These 8 strains all belong to the fermentation type proteus II, which thus appears to be the most penicillin-sensitive type of the proteus bacillus.

Pyocyaneus. Altogether 173 strains were grown, chiefly from urine and otitic pus, respectively 48 and 40 % of the strains. All the pyocyaneus strains are penicillin-resistant, and a great majority of them (86 %) are also resistant to sulfathiazole. Most of them are sensitive to streptomycin. 23 % of the pyocyaneus strains from otitic pus are sensitive to sulfathiazole, but only 6 % of the urinary strains. This fact, that more sulfa-resistant strains of pyocyaneus are found in the urine than in the ear may possibly be due to the circumstance that infections of the urinary tract are treated with sulfa preparations more often than are lesions of the ear, and that several strains in the former affection then become resistant under the treatment.

Alcaligenes. The material includes 20 strains of alcaligenes, cultivated chiefly from specimens of urine and otitic pus. All these strains are penicillin-resistant, and about half of them are sulfa-resistant. Most of the strains are sensitive to streptomycin.

Only 7 strains of *Salmonella* bacteria are examined, namely: 5 paratyphoid B strains, 1 typhoid and 1 *Salmonella enteritidis* Dublin. All of them are penicillin-resistant and streptomycin-sensitive. To sulfathiazole 3 strains are resistant, 4 sensitive.

Pfeiffer bacteria. Altogether 59 strains are examined, 37 of which were isolated from specimens of spinal fluid and 13 from otitic pus. The Pfeiffer bacteria are considerably more sensitive to penicillin than are the other Gram-negative rods. Most of the Pfeiffer strains are sensitive to sulfathiazole, and all of the strains are sensitive to streptomycin, most of them even strongly sensitive.

A considerable part of the Gram-negative rods could not be diagnosed further with the technique here employed. These strains are entered collectively under the designation »Gram-negative rods not further defined«, of which 611 strains are examined. Like the majority of the Gram-negative rods also the greater part of these strains were found in specimens of urine. A few strains in this group have proved somewhat sensitive to penicillin, but a greater majority of them (94 %) are resistant. 68 % of the strains are resistant to sulfathiazole; and most of them are sensitive to streptomycin.

Within the group of *Gram-negative cocci* 12 strains are examined, 6 of which are meningococci, all isolated from specimens of spinal fluid, and all of them sensitive to penicillin, sulfathiazole and streptomycin. The remaining 6 strains of *Gram-negative cocci* are not further defined. They have all been obtained from the respiratory passages. Of these strains 3 are resistant to penicillin, while 3 are sensitive. One strain is resistant to sulfathiazole, while the remaining 5 are strongly sensitive. All the strains are sensitive to streptomycin.

Gram-positive rods. 25 strains of this category are examined, 5 of which are subtilis. These 5 subtilis strains are resistant to penicillin and sensitive to streptomycin; 3 of these strains are resistant to sulfathiazole, 2 sensitive. The remaining *Gram-positive rods* differ greatly in their resistance.

One strain of the fungus *monilia albicans* is resistant to penicillin and sulfathiazole as well as to streptomycin. The same is found to hold good of yeast cells.

A brief survey of the occurrence of the different bacteria.

After this account of the occurrence of the individual bacterial groups and their resistance, it will be appropriate briefly to review which bacteria are found most frequently in infections of the individual organs. In the present material the 2 largest groups of specimens are made up of urine and otitic pus, with respectively 2666 and 1488 strains examined.

The *urines* have yielded quite predominantly *Gram-negative rods* and enterococci (including the enterococcus-like streptococcus), altogether 88 % :

Gram-negative rods	1809 strains = 68 %
Enterococci and enterococcus-like streptococci	522 » = 20 %
Gram-positive cocci (except enterococci and enterococcus-like streptococci)	331 » = 12 %
Gram-positive rods	4 »

Of the 12 % *Gram-positive cocci* 6 % are strains of staphylococcus albus, which most likely represent contamination. Further, no doubt, a majority of the *Gram-positive cocci* not further defined are likewise to be looked upon as contaminating bacteria. Thus there remain but few strains of *Gram-positive cocci* (excluding enterococci and enterococcus-like streptococci) that may be looked upon as cause of urinary infections. As both the *Gram-negative rods* and enterococci are resistant or relatively resistant to penicillin, it is reasonable to expect that penicillin therapy in infection of the urinary tract in most cases will prove ineffective — as indeed has been the general experience.

Pus from infections of the *ear* has mostly yielded *Gram-positive cocci* — namely, 83 % — while 16 % are *Gram-negative rods*. Among the *Gram-positive cocci* the predominant species are staph. aureus,

hemolytic streptococcus and pneumococci (Table 4). Of Gram-negative rods, (excluding the strains not further defined) the pyocyaneus has been demonstrated most often, proteus not infrequently, then the colon group and the Pfeiffer bacteria.

Another of the larger groups of specimens consists of *sputa*, from which 617 strains have been examined. Here, as in the specimens of otitic pus, Gram-positive cocci are preponderant. Among these, non-hemolytic streptococci are the most frequent (220 strains out of 617 = 35 %), then come pneumococci (11 %). *Staph. albus* is found with about the same frequency as pneumococci (Table 4). Of Gram-negative rods, most of the strains belong to the colon group (12 %), i. e., about the same frequency as found for pneumococci. Further, there is found a rather large number of Gram-negative rods not further defined (about 10 %).

In connection with the *sputa* it will be appropriate to mention the *throat cultures*, from which 229 strains are examined, largely isolated from scarlet fever patients, on which account the hemolytic streptococci are the bacteria most frequently demonstrated (112 out of 229 strains = 49 %). Then, in frequency, follow the nonhemolytic streptococci which in throat cultures from non-scarlatinal patients are the most frequent bacteria. 91 % of the strains are Gram-positive cocci and 9 % Gram-negative rods. Among the Gram-negative rods, coli bacteria are demonstrated most frequently (5 %).

From *abscesses* and other forms of pus altogether 221 strains are examined, among which the Gram-positive cocci are by far predominant (184 out of 221 strains = 84 %). The microorganism most frequently encountered here is *staph. aureus* (81 strains). *Staph. albus* is isolated from a not inconsiderable number of specimens (27 strains). 24 strains of enterococci are demonstrated, 16 nonhemolytic streptococci and 13 hemolytic streptococci. 36 strains of Gram-negative rods (16 %) were found in pus.

The next, fairly large, group of specimens is made up of secretions from the *cervix uteri* from which 125 strains are examined: 79 % Gram-positive cocci and 21 % Gram-negative rods. As a rule these secretions contain so few bacteria that these may not be demonstrated with certainty in a direct smear stained after Gram. In most cases no growth is seen on the plates inoculated directly with the secretion, while growth is obtained fairly often in serum broth. So the resistance determination is carried out on this culture, which implies a great risk of contamination — perhaps even as the only bacterial finding. Thus, *staph. albus* has been found to a large extent (39 of 125 strains = 31 %). Leaving *staph. albus* out of account, enterococci are most frequent (19 %), then follow the colon group (16 %), nonhemolytic streptococci (15 %) and *staph. aureus* (6 %). Whether the bacterial strains here found be the cause of the infection present in the indi-

vidual cases, or whether they constitute merely an admixture from the vaginal flora, is impossible to decide.

Pus from fistulas has yielded 106 examined strains. Most of the specimens originated from tuberculous patients. Gram-positive cocci were found in 60 % of the specimens, Gram-negative rods in 40 %. *Staph. aureus* is the microorganism most frequently encountered, then follow *coli* bacteria and enterococci.

In specimens of *spinal fluid* Pfeiffer bacteria are the strains most frequently encountered (37 of 82 strains = 45 %); then follow pneumococci, 15 strains (18 %). Meningococci are demonstrated in 6 specimens of spinal fluid.

Blood cultures have yielded altogether 53 strains, 94 % of which are Gram-positive cocci. Among these strains, non-hemolytic streptococci are the most frequent (31 strains). Only 3 strains are Gram-negative rods: 2 *coli* bacteria and 1 *Salmonella enteritidis* Dublin.

Nasal swabs have given growth of 48 strains, quite predominantly Gram-positive cocci. Hemolytic streptococci are isolated in a large number of cases (23 %) which is explained by the fact that most of the nasal swabs come from scarlatinal patients. Other bacterial strains obtained in this way are non-hemolytic streptococci, *staph. aur.* and pneumococci. From the *peritoneal cavity* 47 strains are grown, 75 % of which are Gram-negative rods. 29 strains (62 %) belong to the colon group, and 4 are enterococci. In 37 specimens of *pus from osteomyelitic processes* 65 % Gram-positive cocci are found, among which *staph. aureus* is the most frequent. Enterococci are demonstrated in 5 specimens.

As to the remaining groups of the material, see Table 4.

Discussion.

Determination of bacterial resistance to penicillin, sulfathiazole and streptomycin is performed after the disc method, which is easy and sufficiently accurate for the present purpose, which has been to get an idea of whether a given bacterium is resistant or sensitive to the antibiotic intended for the treatment of the infection. A higher degree of accuracy is of no importance in this connection. A greater majority of the strains in this material have proved to be either resistant (0) or distinctly sensitive (++) and (+++) to all the 3 remedies mentioned. In practice, the few relatively resistant (+) strains have to be reckoned as resistant, as they may not be expected to be affected by these remedies in ordinary dosage. Possibly larger doses may here prove effective.

The penicillin concentration employed for the disc method is chosen so as to make the observed inhibition zones of suitable size. The concentrations of sulfathiazole and streptomycin are decided on in relation to that of penicillin. Comparison of the diluting method and the

disc method gives an idea of which growth-inhibiting concentrations correspond to the various inhibition zones (Table 1).

Certain difficulties in the sulfa-resistance determination are counteracted to a large extent by employing an ample amount of horse blood (10 %) in the plates and by performing some of the resistance determinations on diluted cultures.

The 5998 strains of the present material originate chiefly from specimens of urine and otitic pus. The bacteria most frequently demonstrated belong to the colon group, enterococci and staph. aureus.

The resistance determinations show that the Gram-positive cocci (with exception of enterococci and enterococcus-like streptococci) on the whole are penicillin-sensitive, whereas the Gram-negative rods (excluding in part the Pfeiffer bacteria) are penicillin-resistant. Most of the Gram-positive cocci (with exception of enterococci and enterococcus-like streptococci) are sensitive to sulfa preparations, whereas most of the Gram-negative rods are resistant to these remedies. Nearly all the Gram-positive cocci (still excluding enterococci and enterococcus-like streptococci) and more than 2/3 of the Gram-negative rods are sensitive to streptomycin (Table 3).

Among the Gram-positive cocci, the enterococci and enterococcus-like streptococci occupy a position by themselves, being rather resistant, in particular, all the strains are sulfa-resistant. Among the pneumococci and the meningococci no strain was found to be resistant to penicillin, sulfathiazole or streptomycin. Nor was any strain of hemolytic streptococci demonstrated to be resistant to penicillin or streptomycin.

It is a striking observation that coli bacteria are distinctly more sensitive to sulfathiazole and streptomycin than are the coliform rods. Among the Gram-negative rods, the Klebsiella bacteria are the most resistant, Pfeiffer bacteria the most sensitive.

The largest group of the present material is made up of specimens of urine (2666 strains). Here Gram-negative rods and enterococci are found to be far predominant, whereas infection with the other Gram-positive cocci is rare. Otitic pus and sputa yield chiefly Gram-positive cocci, even though Gram-negative rods are found not infrequently. Thus, for instance, in sputa strains belonging to the colon group (12 %) are found at about the same frequency as are pneumococci (11 %).

Summary.

Determination of the resistance of 5998 bacterial strains to penicillin, sulfathiazole and streptomycin is performed after the disc method. Results obtained with this method is compared with results obtained after the diluting method.

An account is given of the technique employed, the results obtained and the occurrence of the individual bacteria in the different specimens here examined.

ON GIANT FOLLICULAR LYMPHADENOPATHY

By *Erkki Saxén.*

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In recent literature increasing attention is being attracted by a lymphoid disease known under the name of Brill-Symmers disease, giant follicular lymphadenopathy, follicular lymphoblastoma and follicular reticulosis. The interest aroused by this affection of the lymph nodes is probably due above all to the circumstance that according to the literature, this disease is a clinical and pathologic entity which can appear as independent and benign, but which later often develops into sarcoma, lymphoid leukemia or Hodgkin's disease.

Clinically the disease is characterised by a general or local enlargement of the lymph nodes, often associated with splenic enlargement. A clinical diagnosis is practically impossible to make. The first symptom is usually an enlargement of local lymph nodes, unaccompanied by any feeling of ill-health, and in a later stage giant follicular lymph-adenopathy (g. f. l.) resembles Hodgkin's disease.

It has been found that quite a mild roentgen therapy (300—500 r.) brings about a diminution in the size of the nodes, unless it makes them disappear entirely, but that recurrence is rapid under these circumstances, whereas a stronger radiation (2000—7000 r.) resulted within a longer period in an asymptomatic condition of the patients (Uhlmann, Rubenfeld, Rossier & Spühler).

According to the literature, a microscopic examination of the node is the only way to establishing a diagnosis. Yet, this method also has its limits, which we shall deal with later.

The pathologic anatomy of giant follicular lymphadenopathy (g. f. l.) is known but little, due to the circumstance that in the majority of the cases published the diagnosis has been made only on the basis of one or few node specimens. There is only a scant number of cases in the literature with necropsies performed revealing a similar aspect in all the examined nodes, where the disease therefore actually appeared independently, at least in all published (Terplan, Krause, Symmers).

Histologic changes, which according to the literature are the basis — and the sole basis — of the diagnosis, have however been reported very incompletely, probably due to the circumstance that but little is known of the cells

in the germinal centres and their origin. The only actual change which is reported to have been found in lymph nodes is the »numerical and dimensional hyperplasia of the follicles«, as expressed by the term recurring in the literature. The follicles are round or oval, but their changes are exceedingly variable. They are not only found in the periphery of the nodes but also in their centres, side by side or more isolated, separated by lymphatic or connective tissue. The germinal centres are formed from (1) cells considered embryonic, with a large hypo- or almost achromatic nuclei and a pronounced nuclear membrane, and from (2) similar cells with somewhat darker nuclei, as well as thirdly from (3) large lymphocytes rich in chromatin. These three different cellular types are found in variable correlations, and some follicle can be formed solely from small lymphocytes.

Since it is known that lymphoid structures in general respond to irritation by hyperplasia and numerical and dimensional follicular increase are specially apt to be found in association with infectious states and with neoplasms, the conception seems somewhat strange that a specific disease, giant follicular lymphadenopathy (g. f. l.) can be differentiated only on the basis of small quantitative dissimilarities, a disease which can persist independently or undergo transformation into lymphoid leukemia, lymphosarcoma or Hodgkin's disease.

If this conception is actually true, the disease would present considerable interest in the clarification of the genesis of lymphoid diseases and would at the same time have a clinical significance. It would be particularly interesting to a pathologist who has to produce reports on specimens of tissue sent to him for examination.

Is a pathologist able to maintain, on the basis of those criteria which are established for g. f. l. in the literature, while studying the microscopic picture of lymph nodes, that the disease in question is most serious and frequently an early stage of an affection of lymphoid tissues resulting in death, or should he — as has been the general custom so far — confine himself to a diagnosis of hyperplasia which may develop as a result of very dissimilar irritants?

The material.

In my endeavours to throw some light on the question I have studied 500 specimens of lymph nodes sent to the Patho-Anatomic Department of the University, Helsinki, where the responsible pathologist had made a diagnosis of lymphadenitis, hyperplasia, Hodgkin's disease, lymphosarcoma or lymphoid leukemia. The cases are distributed as follows:

Lymphadenitis chronica	Hyperplasia gl. lymph.	280
Hodgkin's disease	113
Lymphosarcoma	82
Lymphoid leukemia	25

500

Of these I chose those cases which had exhibited changes corresponding to the changes described in the literature in connection with g. f. l. Since however we know the extent of the enlargement and increase of follicles and their germinal centres in different infectious and other diseases, it is natural that

the drawing of a demarcation line for g. f. l. is somewhat arbitrary. Therefore, in choosing the cases, we have endeavoured to make the criteria as strict as possible, and only such cases have been included in which the numerical and dimensional hyperplasia of follicles and their germinal centres was particularly noticeable and striking. Of such cases there remained 15 after a critical examination, 12 belonged to the group Lymphadenitis, Hyperplasia gl. lymph., and in 3 lymphosarcoma was suspected.

Symptomatology.

Now, in all probability, these 15 cases ought to be affected with a special lymphoid disease. Yet it became evident already when studying their clinical picture (table 1), that a lymphoid disease was suspected on the basis of clinical symptoms in less than half of these cases (Hodgkin's disease in 5, lymphoid leukemia in 1 and lymphosarcoma in 1). For the remainder either the following diseases were suspected or the following diseases established: metastatic carcinoma in 5, chronic polyarthritis in 1, dermatitis herpetiformis in 1 and duodenal ulcer in 1.

Histology.

In all these cases, as already mentioned, the removed lymph nodes revealed histological changes similar in so far, that greatly enlarged germinal centres were found in the entire node (fig. 1—3). The original architecture with lymph cords and sinuses had disappeared. The interfollicular tissue was compressed and lymphatic sinuses were generally indistinguishable. There was only a slight proliferation of sinus lining cells and of the medullary reticulum. 8 cases revealed round germinal centres, in 6 some of them were distorted, and in 2 the limiting zone of lymphocytes surrounding the germinal centres was diffuse, with cells of the germinal centres dispersed into the vicinity of the diffuse margin. By the use of silver impregnation methods only relatively few reticulum fibrils were found in the follicles (fig. 4). The reticulum fibrils of the interfollicular tissue were not increased. In general the germinal centres clearly revealed the three cellular types described in the literature.

In order to be able to make a closer comparison between individual cases, nuclei of different types visible in five different fields were counted in five germinal centres of each node. When assessing the result obtained, it must however be taken into account that germinal centres are known to undergo instant changes in alternating active and resting phases. Endeavours have therefore been made to perform the counts from large germinal centres at the place of the largest section. As a basis of classification the grouping already mentioned, usually adherent to in the literature, was used. Cells with a nuclear size of $8 \times 8 \mu$ or more were placed into the first group. The limits between the groups are not clear, but since the writer has himself performed the stainings and made the counts, the figures obtained are of a certain comparative value.

A comparison of the values obtained to each other makes it evident that the variations within the germinal centres of the same node are small, with the exception of case 4. Also when comparing the results obtained from the nodes of different cases one finds that the variations are most insignificant. Yet two cases unmistakably differ from others (6.7).

Discussion.

G. f. l. and lymphoid leukemia.

Case 6 differed from the others by the circumstance that a great majority of the cells in the germinal centres were cells containing

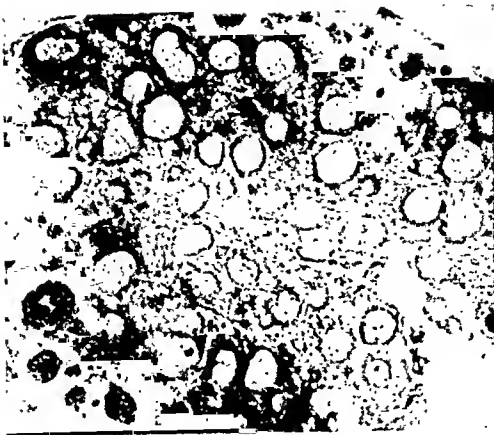


Fig. 1.

Case 8. Clinical diagnosis Dermatitis herpetiformis. The photograph clearly illustrates the numerical and dimensional hyperplasia of the follicles. Van Gieson's Haematoxylin and Pikro-Säurefuchsin.

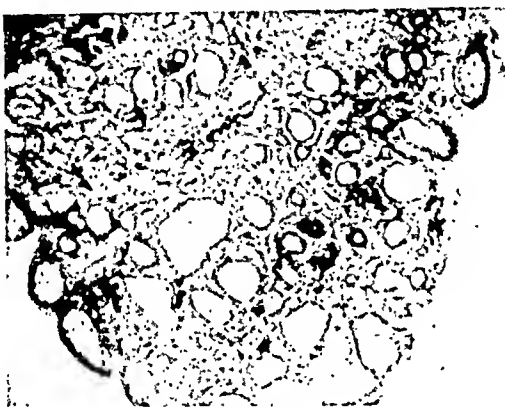


Fig. 2.

Case 10. Diagnosis Carcinoma medullare mammae. The axillary lymph node reveals numerical and dimensional hyperplasia of the follicles. Heidenhain's Iron Haematoxylin.

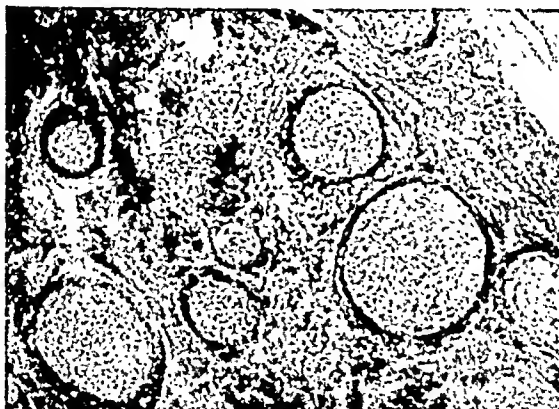


Fig. 3.

Case 1. Clinical diagnosis Hodgkin's disease. Removed cervical node shows a typical g. f. l. picture. Dense zone of small lymphocytes surrounding germinal centres is clearly visible. Van Gieson's Haematoxylin and Pikro-Säurefuchsin $\times 30$.



Fig. 4.

Case 2. Gömöri staining clearly reveals enlarged germinal centres. Reticulum fibres are not increased. Gömöri $\times 220$.

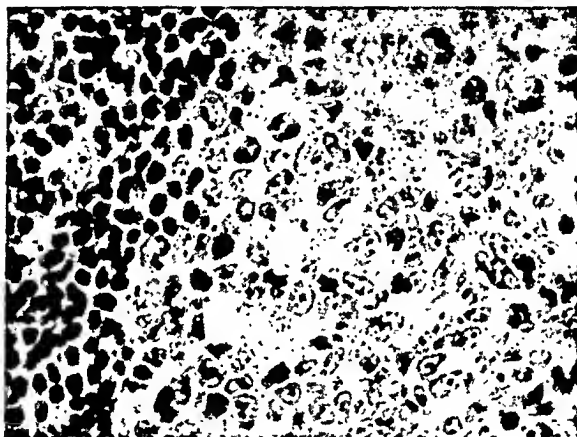


Fig. 5.

Case 5. Clinical diagnosis Polyarthrit. chr. Hodgkin's disease. Lymphocyte zones surrounding germinal centres and their pale nuclei are clearly distinguishable. Heidenhain's Iron Haematoxylin $\times 450$.

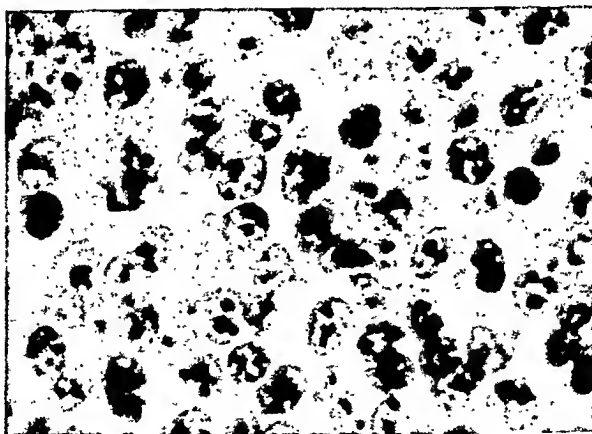


Fig. 6.

Case 6. Clinical diagnosis Leucaemia lymphatica. The germinal centres are formed nearly exclusively from cells with midsized oval nuclei with but small variations in shape and size. The cells are evidently immature lymphocytes. Heidenhain's Iron Haematoxylin $\times 1050$.

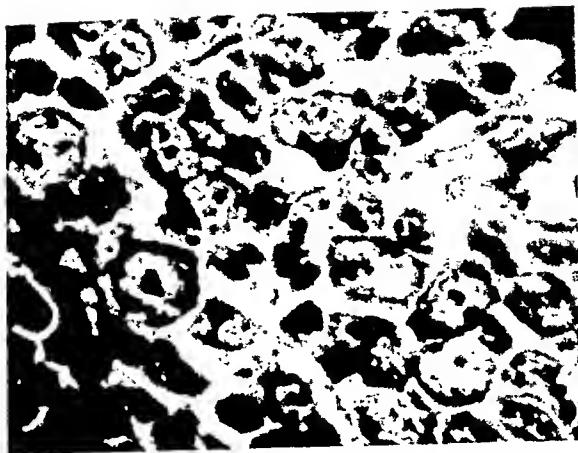


Fig. 7.

Case 7. Diagnosis Lymphosarcoma. Abundant amount of large pale nuclei in germinal centres. Heidenhain's Iron Hematoxylin $\times 1050$.

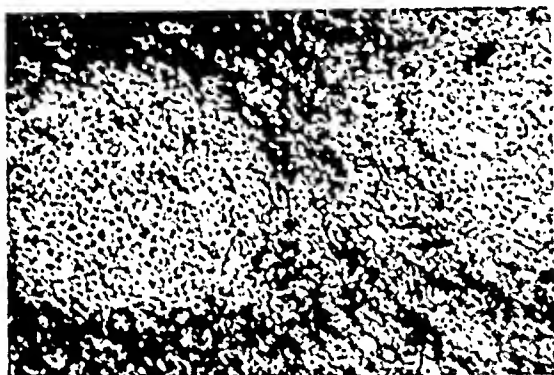


Fig. 8.

Preceding case under weaker magnification. Reveals how the follicles are more diffuse in places and cells of the germinal centres seemingly escaping into the surrounding tissue. Large pale nuclei in germinal centres are visible in this magnification as well. Heidenhain's Iron Hematoxylin $\times 110$.

pale, oval, middle-sized nuclei, with but scant variability in sizes and shapes (fig. 6). The nuclei resembled immature cells of the lymphoid series found in lymphoid leukemia, this disease being here concerned clinically. Even if a clinically pronounced lymphoid leukemia revealed in the nodes changes characteristic of g. f. l. there were yet such dissimilarities in the cells of the germinal centres, that the case is distinctly different from other cases of this material.

Mention is made in the literature that g. f. l. can be »transformed into lymphoid leukemia« (Symmers). However, the circumstance must be taken into account that the cellular proliferation in germinal centres

found in lymphoid leukemia causes swelling and increase of the follicles and the germinal centres, finally filling the entire tissue of the node. Therefore it does not seem surprising that some nodes reveal changes resembling those described in g. f. l.

The case belonging to this series supports this opinion. Of the cases reported in the literature where g. f. l. was transformed into lymphoid leukemia, only two mention the structure of cells in germinal centres (Symmers). In these cases they were chiefly formed from hyperchromatic embryonal cells of the large lymphocytic type. It is therefore possible that there might only be a question of incipient lymphoid leukemia and not of an independent disease g. f. l.

G. f. l. and lymphosarcoma.

Case 7 differs from the others of this series in so far as large pale nuclei were found in its germinal centres to a considerable greater extent than in the others. The case is also individual in such respect, that the same node revealed areas whose germinal centres were not clearly limited, their cells escaping into the surrounding tissue, presenting in places a picture of lymphosarcoma (fig. 7—8).

As already said at the beginning of this paper, mention is made in the literature that g. f. l. can develop into lymphosarcoma. It has been claimed accordingly that nodes can be found in one and the same patient revealing changes typical of g. f. l., and nodes with seemingly ruptured follicles whose cells are diffused into the surrounding tissue, presenting in other words the aspect of lymphosarcoma. It must however be taken into account that these cases may also have been affected with primary lymphosarcomatosis, the g. f. l. changes being secondary, possibly produced under the influence of irritants due to sarcoma. On the other hand, there are cases published in the literature, to which the case in this series also belongs, where regions have been found in the same node with changes corresponding to those of g. f. l., and regions with a picture of lymphosarcoma. It is to be assumed on the basis of these cases that some lymphosarcomas can commence with the enlargement and increase of follicles and their germinal centres. It is noteworthy that the observation has been made in the case belonging to this series, that also those regions which at first glance presented a picture typical of g. f. l., the germinal centres being closely defined, contained considerably greater numbers of large pale nuclei than were revealed in the germinal centres of other cases. It can therefore be assured that at least in some cases the malignity of the disease could be determined with the help of cells in germinal centres. Such an indication was also made by Stout 1947. He classifies the lymph nodes in g. f. l. as belonging to the lymphosarcoma group. He distinguished two different groups. Those where the follicles are clearly limited and hypertrophic in a way corresponding to g. f. l.

and those where the follicles are made up of larger cells and often have diffuse limits. After a period of 5 years his series comprise 50 per cent survivors in the first and 26.3 per cent in the second group. It can be assumed that at least in part of the cases in the former group the lymph node modifications may be due to some other disease, whereas the later is a presarcomatous stage.

G. f. l. and Hodgkin's disease.

Aside from these two cases described above, the histological changes were similar in all instances, i. e. it was not possible to differentiate between them on the basis of the methods employed in the investigation. Of the 15 cases embraced by the material, only 5 remain, i. e. the cases clinically resembling Hodgkin's disease (1—5), which may be a manifestation of the independent disease described in the literature, *g. f. l.* When assessing these cases, it must however be taken into account, that a biopsy was taken in one case only at a later stage of the disease (then simple hyperplasia) (case 4), and only one case (case 5) was examined postmortally (I did not have any histological sections at my disposal). Since only few biopsies were taken or nodes removed at the beginning, it is possible that changes typical of Hodgkin's disease were simultaneously present in the remaining nodes. Nor can it be said with certainty whether the changes remained unchanged until death, or whether possibly a histologically typical Hodgkin's disease developed. In the 3 cases which could be followed up until death, death ensued in 6 months to one year after the removal of the node. It can therefore only be said on the basis of these cases that *g. f. l.* changes in lymph nodes can be found in a malignant disease, clinically resembling Hodgkin's disease.

It has been said already that according to the literature *g. f. l.* can appear as an independent condition, clinically resembling Hodgkin's disease, even if a patho-anatomically typical Hodgkin's disease can develop on the basis of the former. Since, however, in those cases reported in the literature where an also histologically typical Hodgkin's disease developed from *g. f. l.*, the *g. f. l.* diagnosis had been made on the basis of only one or two lymph node specimens the possibility previously referred to should be considered, that these were cases of Hodgkin's disease from the outset and the *g. f. l.* changes secondarily produced by this disease. On the other hand, it cannot be denied that Hodgkin's disease, such as it is described in the literature, could begin by follicular hyperplasia, and that at least in some cases the *g. f. l.* changes could therefore be regarded as an early stage of Hodgkin's disease.

G. f. l. as an independent disease, persisting as such to the final stage, can be demonstrated with certainty only in those infrequent cases published in the literature, which revealed at autopsy in all the lymph nodes examined changes typical solely and exclusively of *g. f. l.*,

Table 1.

Case N:o	Sex.	Age	Clinical diagnosis	Lymph gland swelling at biopsy
1	♀	—	Hodgkin's disease.	cervic. axill.
2	♂	70	Hodgkin's disease.	axill. cubit.
3	♂	20	Hodgkin's disease.	cervic. ingv.
4	♂	43	Hodgkin's disease? Carcinoma intestini? Polyposis intestini?	mesenterial ingv. splenomegaly.
5	♀	25	Hodgkin's disease? Polyarthritis chr.	cervic. axill. splenomegaly.
6	♂	55	Lymphatic leucemia.	cervic. axill. ingv. splenomegaly.
7	♀	52	Lymphoma malignum.	ingv.
8	♀	6	Dermatitis herpetiformis.	cervic. axill. ingv.
9	♀	35	Polyarthritis chr. Anemia gravis.	cervic. axill.
10	♀	44	Carcinoma mammae.	axill.
11	♀	63	Struma maligna.	cervic.
12	♂	55	Carcinoma antebrachii.	axill.
13	♂	56	Carcinoma ventriculi	axill.
14	♀	39	Carcinoma intestini.	mesenterial.
15	♂	45	Stenosis pylori ex ulcere duodeni.	mesenterial.

Duration of l. gl. swelling before biopsy	After biopsy		
	Dead	Living	
—		4 mths.	White blood picture normal. No data concerning later development.
—	1 year		Died from general asthenia.
1 year		6 mths.	White blood count normal.
—	1 year		Abdominal pains during 4 years. Operation revealed a tumour in the duodenojejunal flexure and enlarged mesenteric nodes (specimen 1). Enlarged lymph nodes appeared one year later in the inguinal (specimen 2) and axillary folds. White blood count normal. 2 months later the patient succumbed to general asthenia.
1 mth.	6 mths.		White blood count normal. Subjected to radiation and Ziemsen cure, condition improved, nodes unchanged. 5 months later onset of pericarditis and pleurisy with death ensuing. Autopsy revealed enlarged nodes in the thoracic cavity, abdominal cavity and inguinal and axillary folds.
2 mths.	8 mths.		Blood count revealed 89,000 leucocytes, of them 93 per cent lymphocytes. Subsequent to roentgen therapy and arsenic cure the swellings were absent for 6 months.
10 mths.		1 year	White blood count normal, condition continues good.
2 years		2 years	White blood count normal. Eruption improving sometimes, simultaneously smaller nodes.
1 mth.		4 years	11b. 33. White blood picture normal. Recovered on liver therapy. Developed 3 mths. later severe granulocytopenia (leucocytes 1,000) which responded to blood transfusions and the elimination of aminophenazone the patient had been using for 13 years. Patient is well and lymph nodes have disappeared.
—		4 years	Histologic dgn. carcinoma medullare mammae. White blood count normal.
6 mths.	6 mths.		Cause of death: Dyspnoea compressione laryngis. Atelectasis lob. inf. pulm. Hemoglobin 58, white blood picture normal.
	1 year		Amputatio humeri et evacuatio axillae were performed. Succumbed to general asthenia.
		2 years	Resectio ventriculi was performed. Hist. dgn. adenocarcinoma.
		1 year	Duodenojejunostomia was performed Hist. adenocarcinoma.
		2 years	Gastrojejunostomia was performed. Pains persist. Can manage on a diet.

without any signs indicative of other diseases. Other cases can well be studied also without assuming the existence of an independent disease of the lymphoid system, *g. f. l.*, which in my opinion has not yet been conclusively proved.

G. f. l. and malignant tumours.

Examples of those cases of which it can be said with certainty that they were not an independent disease of the lymphoid system, *g. f. l.*, but which, however, showed similar changes in the lymph nodes as has been described in association with that disease, are provided by the cases of this series in whom the changes in question were revealed in regional lymph nodes removed in connection with tumours or because of suspected tumours (10—14). It should be repeated that the number of follicles was increased, they were enlarged and they were found both in the cortex and the medulla. No significant proliferation of reticular cells were found. The sinuses were narrowed or blocked and the interfollicular tissue compressed (fig. 2). In three cases the primary tumour was revealed by histological examination as carcinoma, and the other two were clinically unmistakable cases of malignant tumours. Not one of them has shown any signs indicating a lymphoid disease. Case 15 may possibly be compared to tumour cases. In this case the lymph node had been removed from the mesenterium in connection with gastrojejunostomy performed on a diagnosis of *stenosis ex ulcere duodeni*. It should be said, however, that this may also have been carcinoma or stenosis brought about by the swelling of the lymph nodes.

All those investigators who have studied the aspect of *g. f. l.* are agreed that the changes in the lymph nodes found in it are difficult to distinguish from hyperplasia associated with neoplasms. According to all the distinction can, however, be made. Thus Heinzelmann writes: »— the diagnosis of *g. f. l.* must be made on the basis that the follicles are larger and more numerous, more densely packed and difficultly distributed in the entire gland — also in the medulla — than in the case of simple hyperplasia.« Baggenstoss and Heck examined biopsied specimens of lymph nodes from 50 cases in which a diagnosis of inflammatory lymphadenopathy had been made and they found that »in none of these cases was there any difficulty in distinguishing the histologic appearance of the nodes from that of follicular lymphoblastoma.«

However, as previously mentioned, the lymph node changes in the 5 cases of this series associated with carcinoma, could not be distinguished from the changes reported in the literature as typical of *g. f. l.*, nor could the distinction be made in the other cases of this material. In other words, it appears as if carcinoma and perhaps also other malignant tumours — probably the tissue necrosis associated with it and different products of disintegration — can produce in regional

lymph nodes changes found in g. f. l. This is also to some extent supported by the observation that hyperplasia found in association with neoplasms has so often been described as »difficult« to distinguish from hyperplasia found in g. f. l.

G. f. l. and skin changes.

Mention is also made in the literature of skin changes appearing in association with g. f. l. (Symmers. Combes). Also in this series there is one such case (case 8). It was that of a girl aged 8 years who for the time of 3 years had suffered from severe eruption (dermatitis herpetiformis). There was a persistent swelling of cervical, inguinal and axillar lymph nodes. According to the history given by the patient, they were smaller while the eruption was better. All three lymph nodes removed presented a picture typical of g. f. l. (fig. 1). This case suggests the idea that severe exudative skin disease can produce g. f. l. changes in superficial lymph nodes, and not that there is a question of an independent disease of the lymphoid system, with associated skin symptoms. This opinion is also supported by the nodes being smaller while the eruption was better. Yet it is true that cases described in the literature, where g. f. l. found together with skin changes developed into lymphosarcoma or into Hodgkin's disease, are difficult to understand on the basis of this assumption.

G. f. l. and polyarthritis.

A special character is exhibited by case 9 of this series, where swellings of lymph nodes were manifested in association with polyarthritis.

This case was that of a woman aged 35, who was affected with chronic polyarthritis, severe anemia and swelling of the cervical and axillary nodes. The Hb was 33, Index 1, 10 and the leucocyte count 8000. The white blood picture normal. Sternal puncture revealed very rapid erythropoiesis and a leucopoiesis slower than normal. A cervical node removed at that stage showed changes typical of g. f. l., with an occasional eosinophil seen among the small lymphocytes in the interfollicular spaces. Anemia responded to liver treatment, but 4 months later the patient developed severe granulocytopenia. The Hb level was 70, Index 0.90, the leucocyte count 1000, transitional 1.5, polynuclear 3.0, eosinophils —, basophils 0.1, monocytes 0.5, lymphocytes 94.0 per cent. Sternal puncture revealed at the time an erythropoiesis of normal velocity and a normal amount of lymphatic cells but granulocytic elements were entirely absent, with the exception of basophilic leucocytes. The anemia improved subsequent to blood transfusion and to the elimination of aminophenazone which the patient had been using for 13 years. After this the patient has felt well for a time of 4 years. Nor could any swellings of the lymph nodes be found later.

In case 5 as well the patient had suffered from chronic rheumatic polyarthritis during 6 years prior to the onset of a clinically typical Hodgkin's disease. This case has been dealt with in connection with Hodgkin's disease.

There are at least three cases described in the literature (Symmers, Heinzelmann, Chevalier) where swelling of the lymph nodes was associated with polyarthritis and where the structure of the lymph nodes was that manifested in g. f. 1. Of these, the case described by Symmers 1938 is practically similar to case 5 published in this paper. Special features were the necrotic follicular changes observed in the lymph nodes after death. In Heinzelmann's case, on the other hand, the histological changes resembled more simple hyperplasia which can be seen in association with polyarthritis. Chevalier's case can perhaps be compared to case 9 of this series. In his patient the causative agent was evidently a toxic gold dose, although possibly transmitted by dermatitis. In the case belonging to this series one is tempted to assume that the aminophenazone might have played a share in producing swelling of lymph nodes.

Summary and conclusions.

The material comprises 15 cases collected in such a way that of 500 lymph node specimens diagnosed by the examining pathologist as lymphadenitis, hyperplasia, Hodgkin's disease, lymphosarcoma or lymphoid leukemia, such instances were chosen whose structure conformed to the criteria put forward in the literature for giant follicular lymphadenopathy. Of these 12 were diagnosed as lymphadenitis or hyperplasia, and in 3 lymphosarcoma was suspected.

While studying the clinical picture of the cases, it became evident that the clinical aspect corresponding to similar changes in the lymph nodes was of a vastly different nature. Similar changes were found in association with carcinoma in regional lymph nodes, in superficial lymph nodes associated with exudative skin disease, and in patients affected with chronic polyarthritis. Changes of similar nature were also correlated with lymphoid leukemia, lymphosarcoma and a clinically typical Hodgkin's disease.

Hyperplasia manifested in lymphoid leukemia and lymphosarcoma could be distinguished from hyperplasia found in the remaining cases of the series, by means of differences seen in the nuclei of germinal centres, and by the circumstance that in places the cells seemed to escape into the surrounding tissue.

In the lymphoid leukemia the germinal centres consisted exclusively of cells with oval, middle-sized nuclei, with but small variations in shape and size, cells which must be regarded as juvenile forms characteristic of cellular infiltration in lymphoid leukemia.

In lymphosarcoma the germinal centres revealed abundant amounts of large pale nuclei.

The etiological factor was probably the tissue necrosis and different products of disintegration in hyperplasia manifested in connection with carcinoma and exudative skin disease. In the case affected with

polyarthrititis it is possible that aminophenazone had been responsible for the swelling of the lymph nodes. Hyperplasia found in lymphoid leukemia and lymphosarcoma must evidently be regarded only as an early manifestation of the disease.

Of the 15 cases comprised by the material, an independent disease of the lymphoid system, g. f. l. can only be entertained in those 5 cases who had a clinical resemblance to Hodgkin's disease. Yet, where these cases are concerned, the possibility is equally probable that they were barely instances of incipient Hodgkin's disease or that the changes characteristic of g. f. l. were a secondary development in regional lymph nodes under the influence of »toxic agents« associated with Hodgkin's disease. In other words, the genesis would be similar to that found in hyperplasia in connection with carcinoma.

Although the drawing of conclusions is greatly limited by the circumstance that only a few lymph nodes had been studied in these cases, it can yet be claimed with certainty that similar changes as reported in the literature in connection with g. f. l., can be found and often are found without there being any reason to assume the presence of an independent disease of the lymphoid system.

In cases where g. f. l. has been described as preceding lymphoid leukemia or lymphosarcoma, it is possible that the disease was incipient lymphoid leukemia or incipient lymphosarcoma. A distinction between them and other follicular hyperplasias may be possible on the basis of the nuclear dissimilarities found in germinal centres prior to any changes in the interstitial tissue.

It should be said that the diagnosis of an independent disease of the lymphoid system g. f. l., which according to the literature is to be made solely on the basis of a histologic examination of lymph nodes, is not only limited by the fact that the same node can reveal areas of different structures and the same patient have nodes of a different structure, but also by the fact that changes associated with g. f. l. can be found in numerous other diseases.

These limitations of the histologic studies also reduce the value of observations made in radiation treatment of g. f. l., since in these cases the diagnosis is generally established only on the basis of one or two lymph node specimens.

The changes manifested in g. f. l. are not specific this being evidently a question of only some mode of reaction of the lymphoid system to several different toxic and/or inflammatory agents.

The observations made on the basis of this material perhaps justify the conclusion, important in a practical sense, that if we see a histologic picture of g. f. l. revealed in some lymph node specimens, we have reason to suspect a serious condition. The material evidences furthermore that this disease can be of very dissimilar nature, being only in some cases a true disease of the lymphoid system. The observations made in this investigation also indicate that by making an exact dif-

ferentiation of the dissimilar cellular shapes of enlarged germinal centres, better results can be obtained in diagnosis. Such conditions as lymphoid leukemia and lymphosarcoma could then perhaps be distinguished from other diseases where numerical and dimensional hyperplasia of the follicles is found.

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STUDIES OF THE LIFE-CYCLE OF PROTEUS HAUSER

PART 2

By Johs. Kvittingen.

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In a previous work, published in this journal, it was shown that *Proteus Hauser* has a life-cycle, which was broadly accounted for. It was established that regularly arranged granules are formed in the »swarmers« during the rest-period, and that the division takes place through the middle of these, so that each new cell gets a granule in either end. The division also takes place through the terminal granules. The rather strange phenomenon was pointed out, that the end-pieces that are partitioned off, get only half the granular mass of the other segments. The fate of these »monogranular« end-pieces was discussed, and it was suggested that they in some way or other stood in relation to the development of the swarmers.

To study the cellular development in greater detail and possibly find out the mechanism and development of the swarmers, experiments were made with the intention of isolating single cells or groups of a few cells and watch their development. Different phases of the cycle described were taken as starting-points for these studies, and on the following pages an account is presented of:

1. The cellular development from short forms to swarmers.
2. The cellular development from swarmers in different stages via short cellular forms (multiplying forms) to development of new swarmers.

Media, inoculum and technique.

1½ % agar plates were used with cast-in slides as before described. The agar layer over the slide was measured to about 1 mm, and the plates were inoculated as soon as they had congealed without being dried in the incubator.

As inoculum was used a suspension of microbes in the phase wanted as starting-point. A drop of the suspension was spread over the agar plate with a bent glass-rod. By help of the drylens of the microscope, the plate was inspected at once, and in case the inoculum was too dense, an adequate portion of microbes was removed with the rod.

The slide was cut out and placed on the mechanical stage of the microscope. Over a field of suitable density of microbes was placed a cover-glass, the agar outside the cover was cut off and the edges were sealed with paraffin to prevent drying during the experimental period.

The inoculum was taken from a plate culture, which at any time could be observed under the microscope. By help of direct inspection of the live culture, and, if necessary, supplemented by stained preparations, it was quite simple to choose parts of the plate with cells in the phase wanted.

In some experiments the slide was cut out as soon as the inoculum had been spread. In parallel experiments the slide was cut out after some time's incubation, at a stage when it could be seen that the cells were in lively division.

The experiments were made both at room temperature and at 37° C. The slow development at low temperature no doubt gives the best insight into what is happening. But on the other hand, it binds the observer and apparatus for a long time. To speed up development the experiments were, as mentioned, partly carried on in incubator at 37° C. The microscope was then placed in the incubator.

For technical reasons and partly for lack of films, it has not been possible to supply this work with as many and good photos as desired. In some cases sketches had to be made.

1. *The cellular development from short forms to swarmers.*

With the technique described above, an inoculum, that contained practically only short cell forms, was spread. After repeated observations of the development, both of a single cell and of small groups of up to 4 cells, from the moment the inoculum was spread, till the moment the new-formed swarmers made their first movements, it was possible to form an opinion of what was happening. First an attempt will be made to describe the development without relation to time or special experiments, and afterwards some actual observations will be reported.

The short cell forms pass through: 1, a multiplying phase, and 2, a differentiation phase.

To 1. In this phase the cells divide into two seemingly identical individuals. It is possible to ascertain that the newly partitioned cells have a compact body in either end, that the same cells, when they in their turn have come to their division period, have got a compact body in the middle between the terminal bodies, and that division takes place through this one.

To 2. After a greater or smaller number of divisions, it appears that some cells take quite a peculiar course of development. Instead of dividing in the middle into two quite identical individuals as before, they abscind a short piece at one end. After the abscission it looks as

if in some cases, the cell has finished its multiplying period, in a relatively short time growing into a swarmer. The abscinded end-piece does not divide and grow, as long as it is possible to keep it under observation, that is till the swarming begins. Whether the end-piece is a Cell-part capable and living, or a part that will disintegrate, is difficult to decide, because of the short time it is possible to keep it under observation

It is difficult to ascertain how large a part of the short cell forms is transformed to swarmers in the way described above. For as the cell group increases, the observation becomes more difficult, and when the number has reached 30—40, it is next to impossible to keep account of what is going on. But it looks as if most, possibly all, cell forms, under favourable conditions after a period of multiplication, develop into swarmers. As far as it has been possible to observe this development, it seems to be tolerably certain that only a smaller number of cells abscind the end-piece before they change into swarmers.

As mentioned before, it is very difficult to get a full survey of what is going on in a somewhat large cell group. A picture of the cells of a group, can however, be got in the moment the swarmers are beginning to move. It can then appear that in some groups practically all cells are more or less fully developed swarmers. In other groups there may be only a few swarmers that are beginning to move, while the rest still is short cell forms in intense bi-partition. But gradually fewer and fewer short cell forms are to be found, while the number of swarmers seems to increase correspondingly.

A very interesting group that was observed, will be described in detail at several stages of development, and supplemented with sketches. The preparation was made from a plate that had been left standing at room-temperature after inoculation so long, that the cells had divided several times and formed larger and smaller groups. At this moment the slide was cut out and placed on the mechanical stage of the microscope. A group of medium size was placed under the microscope, and by putting on a cover-glass, it was scattered into several groups. One of these consisted of 3 cells, one of which showed a more compact body uttermost at one end. The following development was observed under the microscope in the incubator at 37° C.

At 21.00: Microscope with preparation placed in incubator at 37° C.

All 3 rods are of about 3—4 my's length. One rod has a granule in one end.



21.10: The microbe with a granule in one end has begun to abscind it.



21.10: The granule mentioned is quite abscinded. It has a little



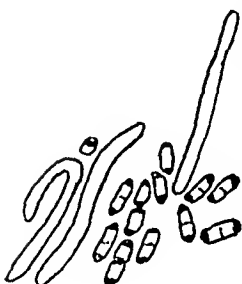
protoplasma around it, and is now lying beside the rest of the rod. One of the other rods of the group has begun to divide in the middle.



21.35: The microbe that abscinded the monogranular end-piece, has increased greatly in length, looks quite homogenous, and does not show any indication of division. The abscinded end-piece has perhaps grown slightly. The other two microbes of the group now have divided into 4 short forms.



22.10: The group now consists of a large swarmer, which still has not moved. The abscinded end-piece is unchanged. The other two have now multiplied to 12 short rods.



22.55: Several of the short rods are dividing. The abscinded end-piece is unchanged. At this moment 2 swarmers from neighbouring fields come into view. One comes bent, and with the bend foremost, and places itself beside the swarmer in the group observed. A few moments later this swarmer makes some very quick strokes and jerks, so that it sweeps all short rods in its group, to all sides. Swarmers now invade the field from all directions, and individual microbes cannot any longer be accounted for.

Form and size of the abscinded end-pieces.

Form and size of these cell fragments vary somewhat. Often it looks as if there is a spherical body, that seems to consist of only a dense mass. More rarely the abscinded part is extended, and consists of a compact body embodied in some protoplasm. Photographical reproduction can sometimes give a misleading picture of the end-pieces. An extended piece may, in the moment it is abscinded, place itself on edge, and will be projected on the negative as a round body. In the drawn series the end-piece was distinctly longer than the diameter of the cell, and in Photogroup 1 one spherical and one long end-piece are clearly visible. This difference of form has probably no significance for the later fate of the cell formations, as neither the spherical, nor the long forms develop further or change in any way, as long as they can be observed.

An important question arises in connection with the development of short cell forms into swarmers. What biological conditions decide

the change of phases? Many factors can, no doubt, be drawn into the discussion here. On the basis of the observations that are made, it is, however, impossible to draw definite conclusions, and therefore it must suffice to discuss the most likely suppositions:

1. Does the transition into swarmers come after a fixed number of divisions?
2. Is it the stimulus of bio-chemical changes in the milieu that gives the impulse to the development of swarmers?
3. Or is it, as supposed by some, toxical substance in the milieu that cause the change of phases?

It should be theoretically possible to test the first possibility experimentally, if sufficient technical equipment is available and if the experimenter has enough time and patience. The inoculum must in that case consist of short cells from a hunger-milieu, which had stopped their multiplication, or of undivided swarmers. The best insight into the multiplying phase is to be had, when the inoculum consists of short forms, as it then is possible to start with one cell, and the group therefore will be comparatively small and surveyable at the moment, when differentiation into swarmers begins. The weakness of this method is that there will always be a certain obscurity about the conditions of the cell and its relation to the multiplying phase at the start. The biological condition of the organisms should be more constant, if the development from the primary segments of a swarmer is studied. The inoculum must then consist of undivided swarmers, and by mechanical means a segment must be isolated, as soon as it is abscinded. The isolation of a single segment is, however, no easy matter, and in case the experiment begins with a whole swarmer, other complications may arise. It may be very difficult to decide with certainty how many primary segments a swarmer divides into, because single segments may begin to multiply before the primary division of the swarmer has finished. Even if it therefore was possible to ascertain the number of new swarmers that descended from »the individual experimented on«, in this case a whole swarmer, it would nevertheless not be possible to reach any conclusive answer to the question whether change of phase from short cell forms to swarmers occurs after a fixed number of divisions. Multiplication from all the segments of a swarmer leads also to such large groups that they are rather unsurveyable.

The observations made in this work do not give a conclusive answer to the first question. They suggest though, that it is not a fixed number that precedes the changes of phase. It is more probable that the cause must be sought in stimulating bio-chemical changes of milieu. A fact that points to such conditions as cause of change of phase, is that on a favourable moist medium, where the cell-division is much slower, the groups as a rule grow much larger before differentiation into swarmers begins.

As mentioned in Part I of this work, most of those who have studied *Proteus* before, reached the conclusion that change of phase from short cell forms to swarmer is caused by toxic changes of milieu. It was suggested that such an explanation hardly is tenable. As above mentioned, it appears that in a favourable milieu great masses of swarmer are quickly developed, and the swarmer develop in several relays with a multiplying phase of short cells between the relays. In proportion, as the conditions of life must be supposed to deteriorate, (among other things because of accumulation of toxic substance in the milieu) fewer and fewer swarmer are developed, and most of them do not succeed in coming through a normal development, but change into morphologically very abnormal forms.

Technical difficulties and lack of photographic equipment are the main reasons why it has not been possible to support this work with photographic reproductions to the extent that is to be desired. The object-matter of these studies was live organisms that changed morphologically from minute to minute. As an example may be mentioned that a stage which it seemed desirable to photograph, as a rule was passed, in the moment everything was ready for photographing. Ideal conditions for work would demand that there are, at any time, sufficient films and microphotographic equipment available, in a suitably heated room, so that a valuable find immediately could be recorded on a negative.

With the facilities that have been at disposition an attempt has been made to observe the multiplying phase of the short cells with photographic reproductions. Some situations from the development of 3 different groups will be given, and it is hoped they will support the written account of the cellular development.

GROUP SERIES 1. Fig. 1—6.

The inoculum consisted of swarmer which had quieted down and more or less had finished their primary division. The further development took place at room-temperature (ab. 20° C.). Group 1 consisted in the moment it was placed under the microscope, of 3 cells, which were lying in the centre of the field of vision. Two of these were relatively short. The third one, which was longer, had a nearly abscinded »end-piece«. To both sides of the central group lay a quite long swarmer. It is probable that the central group represented a divided swarmer.

Fig. 1 shows the situation about 1 hour after the preparation had been made. The central group has increased to 6 cells, and a completely abscinded round »end-piece« is laying close to the group. The cell to the right in the field of vision, is abscinding a more elongated piece of its upper end. The cell at the left is unchanged.

Fig. 2 shows the situation 1 hour later. The central group now consists of 8 cells, and the round »end-piece« is to be found to the right,

Group Series I.
Magn. about 1450 X.

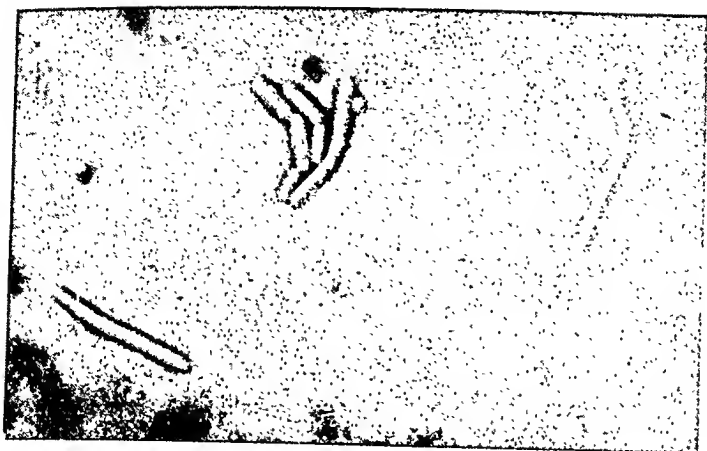


Fig. 1.
1. hrs. 13.25.

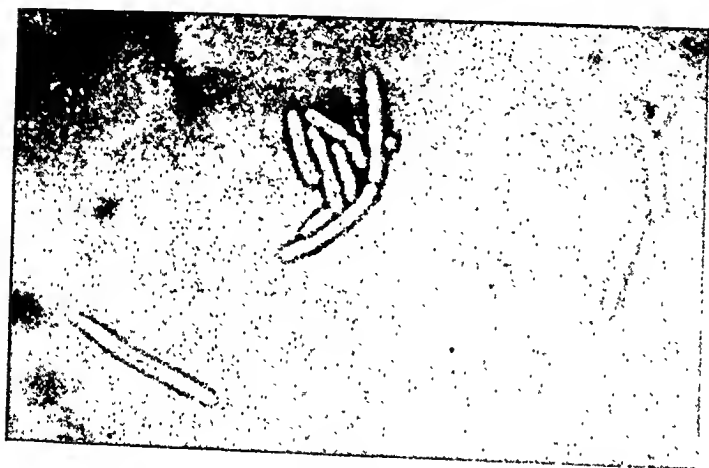


Fig. 2.
2. hrs. 14.25.

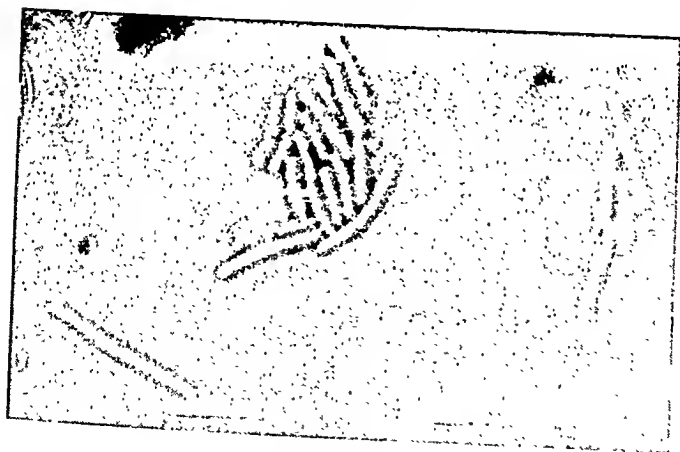


Fig. 3.
3. hrs. 15.50.



Fig. 2
2 hrs 1945



Fig. 3
3 hrs 1945

close to the group. To the right in the field of vision, is found that the extended "end-piece" is quite absconded and laying a little further down forming an acute angle with the cell, which has divided in the middle.

Fig. 3 shows the situation 1 hour 25 min. later. The central group now numbers 15 cells and at least 4 of them have got rather far in the progress of dividing anew. The round "end-piece" is still unchanged, and at the right uppermost in the group, a cell is absconding

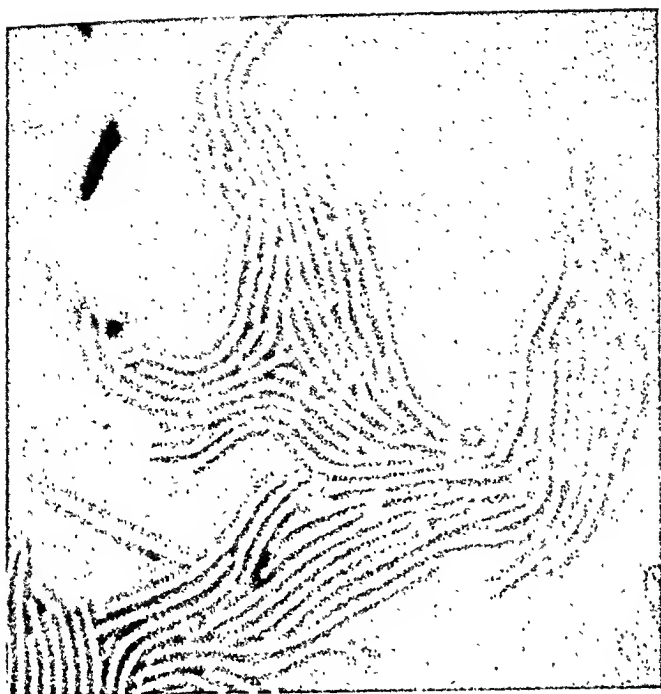


Fig. 6.
6. hrs. 19.05.

a rather long »end-piece«. The group to the right in the field of vision numbers now 4 cells, of which 2 are nearly divided. Neither has the »end-piece« belonging to this group changed in any way.

Fig. 4 shows the situation 1 hour 50 min. later. The central group numbers ab. 25 cells, and some of these are dividing. One sees, however, that at this moment many of the individuals have grown considerably longer, which means that they have stopped their multiplying phase and have begun to develop into swarmer. The »end-pieces« are still found unchanged, the round one just inside the group, closed in by the long cell to the right. The group to the right in the field of vision numbers 10 cells, of which most have begun to grow in length. The »end-piece« belonging to this group is also still unchanged.

Fig. 5 shows the situation 1 hour 5 minutes later. The central group has grown considerably in extent, — mostly because most of the individual cells have become quite long swarmer, while the number of individuals has increased only slightly during the last hour. The group to the right has developed parallelly with the central group, — possibly with the difference that the swarmer development has not gone quite as far for the greater part of the individuals. The »end-pieces« are found in both groups and are still unchanged. The picture shows a swarmer development which has got so far that it is to be

expected at anytime, that some individuals will begin to move. About 15 minutes later swarmers from other groups began to stream into the field of vision at the same time, as some individuals in the central group began to move.

Fig. 6 shows the situation 20 min. later. The swarmers from the environment have got contact with the observed groups, while it also shows that some individuals of the central group have moved. Nethermost to the left, a swarmer is on its way out of the group, and by the upper pole two are on their way out. None of the swarmers of the right group have yet moved. The »end-pieces« which partly have been followed from *Fig. 1*, have not given any indication of growing or developing in any way, or any indication of quickly decomposing are seen for the last time at this stage. Some minutes later, the field of vision is full of swarmers in one seething mass, and there is no possibility of observing any definite object.

The comparatively long swarmer to the left in the field of vision is found again in unchanged state in all figures. Similar cells, which do not show any indication of development, nor any visible signs of degeneration in the course of the observation period, have been observed several times.

GROUP SERIES 2. FIG. 1—3.

This series is from the same preparation as Group 1. *Fig. 1* shows the group situation at a moment ab. 15 min before *Fig. 1*, group 1. The group numbers 4 cells of different length, and 2 of them are abscinding »end-pieces«, the lower one round of form, the upper one extended and fusiform. It is possible that here is a swarmer in its primary division, and that the 2 »end-pieces« are the formerly described monogranular segments from the swarmer's both ends.

Fig. 2 shows the the situation 1 hour 30min. later. The number of cells has increased to 15, and several of them are dividing. The »end-pieces« are found at the upper and nether poles, unchanged.

Fig. 3 shows the situation 3 hours 20 min. later. The group has reached a considerable size, and by the magnification that was used, it filled the greater part of the field of vision. To count the individuals in such a large group is very difficult, not to say impossible, and what happens to the individual cells in this society, is still further away from what can be observed by eye. Even a far from first class film reproduction, gives a better survey than direct observation. From this figure it is possible to establish that the number of cells has increased to about 60, and many — perhaps the greater part — of these are still in their multiplying phase. Especially along the left flank of the group, although also in other places, are found some cells which have a more homogeneous appearance and have begun to stretch lengthwise, so that some individuals have already begun their development into swarmers. The »end-pieces« which were abscinding in *Fig. 1*, are

Group Series 2.
Magn. about 1450 X.



Fig. 1.
1. hrs. 13.10.

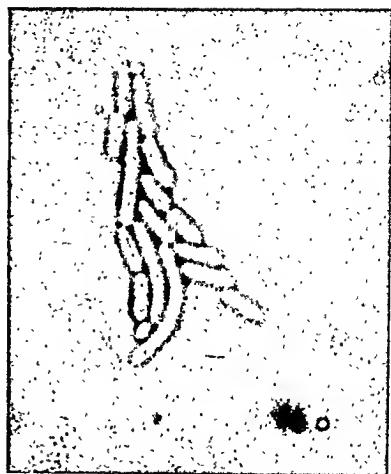


Fig. 2.
2. hrs. 14.40.

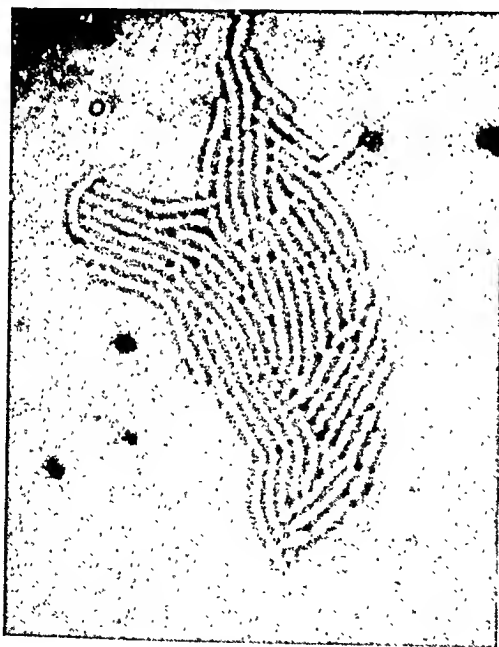


Fig. 3.
3. hrs. 18.00.

found unchanged, even at this stage. The upper one is lying just within the edge of the group, and the nether one is quite free, at the nether pole of the group. Here it looks as if there should be two round bodies, but one of them is an artifact.

GROUP SERIES 3. 1—3.

The inoculum is taken from a plate culture, from a field where the swarmers had finished their primary division, and the individual segments had started their multiplying phase. *Fig. 1* shows the situation after the preparation had lain some hours at ab. 18° C. For the most part there still are short cells that are in their multiplying phase, but also some young swarmers are to be seen. Between the groups there is a long and very thick, nearly fusiform cell, which looks quite homogeneous in its structure. At the upper end is a just abseinded round »end-piece«.

Fig. 2 shows the situation 2 hours 35 min. later. What is first noticed, is that the large, fusiform cell which was lying between the groups, is gone. It suddenly began to move and swam quietly out of the field of vision. But the »end-piece«, which it had abseinded, remains at about the same place it had in *fig. 1*. Further is noticed that a larger number of cells have begun their differentiation into swarmers, and about 2 hours later the field of vision was overflowed by swarmers in lively movement. The preparation remained under the microscope, and *Fig. 3* shows the same field of vision some 17 hours later. The picture is as commonly seen when conditions of life are not optimal. Drying and accumulation of toxic stuff have so greatly influenced the development, that only a small number of swarmers have succeeded in making a normal division. One part of the swarmers is lying, morphologically unchanged, without any sign of division, and an even larger number is changed into clubs, balls and other abnormal forms.

THE DEVELOPMENT OF THE SWARMERS FROM DIFFERENT »STAGES OF RIPENING« TO NEW SWARMERS

The eyele from divisionable swarmers via short multiplying forms, has already in the main been described. But during the work with swarmers as inoculum, other things turned up, which deserve to be mentioned especially and more elaborately.

With the technique described above, a suspension of swarmers was laid out on agar plates with cast-in slides. The plates were inspected as soon as the inoculum was laid out. It then appeared that in some experiments, the swarmers at once quietened down, or soon after having come into their new milieu, whereas they, in other experiments continued to swarm about. Further, it was striking that in the cases, when the cells quietened down at once, it seemed as if most individuals had the ability to divide and multiply as usual, in the way described. It was somewhat different in the cases when the cells of the inoculum continued to move after they had been spread on a new agar plate. Then the rather astonishing fact appeared, that only

Group Series 3.

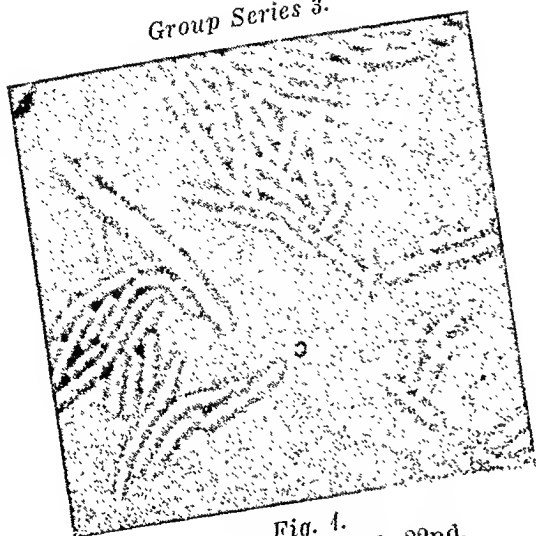


Fig. 1.
1. hrs. 16.35. March 22nd.

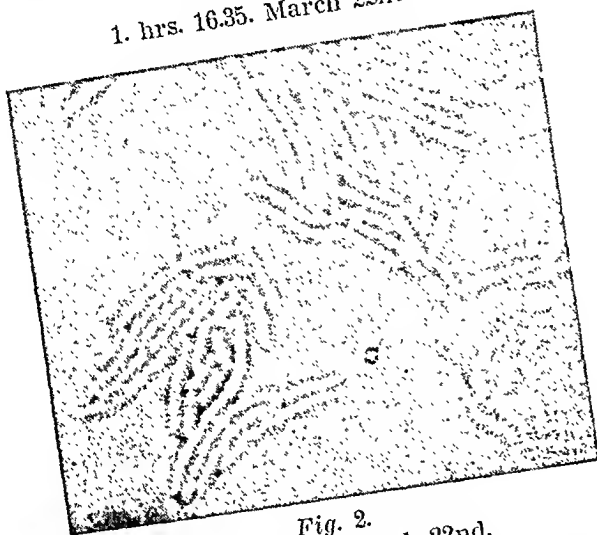


Fig. 2.
2. hrs. 17.25. March 22nd.

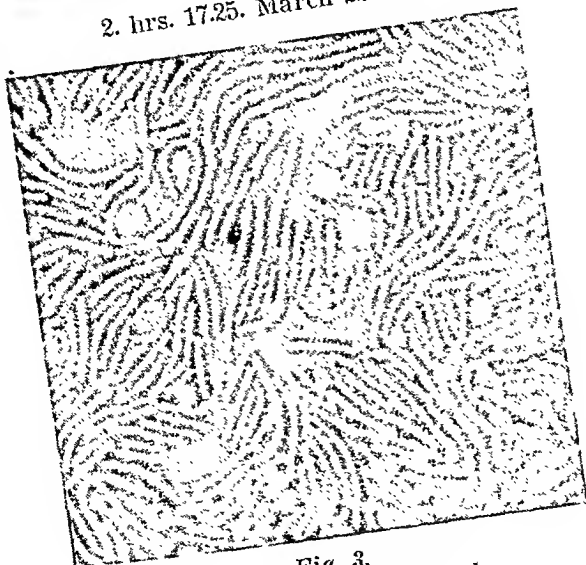


Fig. 3.
3. hrs. 10.00. March 23rd.

part of the transplanted individuals — when they finally had quietened down — divided and continued to live on, whereas the rest comparatively quickly decayed.

This phenomena was at first quite confusing. Firstly, it was strange that it had not been noted before, and secondly, it put a questionmark to the explanation of some of the previous observations.

The first conceivable explanation of this problem was, that the swarmers come into being, develop and thrive in a biochemical milieu prepared by their own colony, and that only a minority survives the stress, which the transplanting into a strange, not thus prepared milieu represents. But not quite everything fits this explanation, for as already mentioned, in some experiments the majority of the transplanted swarmers decayed, whereas in others the majority was able to divide and live on. Partly the cause of the different fate of the cells therefore must be due to biological conditions in the individual itself. With these new finds in view, it fell natural to take a retrospective look at some of the finds that were made formerly, but which had been deemed of no account.

During the first work with the life-cycle of the microbe, impression preparations were used to a large extent. In these were often found, badly stained, thin and shabby swarmers among the strongly stained and granulated ones. Such finds did not then cause any suspicion that there was anything special to be taken into consideration, and it was without questioning accepted as reasonable, that some of the swarmers disintegrated. On basis of the new finds, the question was raised whether it possibly might be a normal phenomena, that a larger or smaller part of the swarmers disintegrated, even if they lived in their natural milieu. These questions necessitated a renewed and closer study of the swarmers.

As already mentioned, it was natural to point to milieu conditions and some experiments. Because, as shown before, e. g. the degree of humidity is of the greatest importance for the development of the swarmers. Some parallel experiments with media of varying degree of humidity were carried out. The results indicated that changes of milieu hardly could be the primary cause of the different fate of the individual swarmers. Admittedly, it was a fact, that in case the inoculum contained swarmers which continued to swarm in the new milieu, then the movements were freer and livelier on the moistest media. But in some cases the swarmers, after the transplantation, quite soon, or at once, quietened down on all media, and in the cases, when this happened, most of the cells on all media, seemed to have the ability to live on.

The inoculum was taken from swarming plate-cultures. A drop of broth was put in the place from where the swarmers should be taken. After very careful stirring, a small mass was transferred to the new medium — there mixed with a larger drop of broth and then

spread with a bent glass-rod. At first the inoculum was taken from some chance place in the swarming one. An inoculum taken from the edge of a swarming colony contains some cells which in any case, are in movement at the moment it is taken. But as a rule these cells quietened down as soon as they had come onto the new medium. In inoculum taken from a more central part of the swarming one, most often some of the swarmer continued romping about in the new milieu. This indicates, that perhaps it is the stage of development of the swarmer, that is decisive for whether they should live on or decay after the transplantation. To test this supposition, it was experimented with swarmer at different stages of development.

By help of a microscopic inspection of the uttermost zone in the rest-phase of a plate-culture, it is easy to ascertain when a new generation of swarmer begins to move. First they begin quite centrally in a zone, and like a wave, the teeming life is spreading, and at last passes across the border of the colony, thereby starting the beginning of a new zone.

If the inoculum is taken from the central zone, at a point of time just after the swarmer have begun to move, then it appears that all the cells continue to swim around in the new milieu and then pass to quieten down in body or singly, and further it appears that it is only a minority of the individuals under observation in the field of vision, that divides and multiplies. The majority withers and decomposes.

The decomposition happens comparatively very quickly, but like other biological phenomena, in this cycle depends on temperature and medium. At 37° C. and in a milieu where the development is quick, it seems to take only a few minutes, from the moment a swarmer quiets down, till it is dead and more or less decomposed. If this process is placed in relation to the development of individuals capable of living, it practically covers the situation to say, that when the latter swarmer have finished their primary division, then, as a rule, the other category may appear as shadows or they have completely disappeared. The astonishing quickness of the disintegration explains the fact, that the phenomenon has been overlooked.

It is difficult to have any definite opinion of how large a part of the swarmer disintegrates. The finds vary seemingly in close relation to the stage of development of the swarmer in the observation material. If the material is taken at a very early stage, so that it can be ascertained that a new generation of swarmer just then begins to move, then it looks as if the majority of cells only has a short life-time or perhaps more correctly, swarming-time, and then disintegrates. Is the inoculum, however, taken at a later stage of development of the swarmer, then the ratio between those capable of living and those that decay, has changed in favour of the first category. As will be shown later, it must be supposed that the cause of this change simply

lies in the fact, that some of the cells not capable of living, already have disintegrated.

In support of the exposition, two actual observations shall be referred:

1. Inoculum with young swarmer. When these had quietened down, a field of vision of 19 cells was fixed and their observation continued. 17 individuals decayed — and only 2 divided into short segments, which continued the usual multiplying phase.

2. In this experiment swarmer in a later stage of development were plated.

The preparation was inoculated at hrs. 13.45 and all the swarmer soon quietened down. The preparation was held at room-temperature till hrs. 15.00, when a field of vision of 39 cells was chosen for further observation. After one hour's incubation at 37° C. there were within vision, 23 cells showing a comparatively normal appearance, and also there were 9 barely visible ghost cells. The other 7 were completely decomposed. After still an hours continued incubation at 37° C. there still were 23 cells in vision. 14 of these looked normal and most of them were dividing. 9 cells had changed into head forms.

The two experiments should show, what difference there is between cells capable of living and cells not capable of living in inocula which represent a very early stage in the swarmer phase, respectively.

Some photos have also been taken, and it is to be hoped that the reproduction is so clear, that they show part of what has been described.

GROUP SERIES 4. FIG. 1—3.

Fig. 1 shows the situation shortly after the young swarmer have quietened down. The cells capable of living are compact and give a far better contrast to the medium, than the ghost cells, which are in majority.

Fig. 2 shows the situation 6 hours later. The development has taken place at room-temperature. (17° C.) Among the ghost cells are seen the cells capable of living. Some have not quite finished their primary division, whereas others have begun multiplying.

Fig. 3 shows a phenol-fuchsin stained impression preparation which corresponds to *fig. 1*. The difference between cells capable of living and ghost-cells, is marked.

Morphologically there is little difference between the quite young swarmer. Sometimes individual cells can be seen, which are considerably larger than the average, others appear more compact, and in some can be seen intracellular, more compact bodies, which often are arranged like pearls, along the microbe. These finds are, however, not so constant and characteristic that it is possible, on that basis, e.g.

Group Series 4.



Fig. 1.
1. hrs. 13.30. Magn. 650 \times .

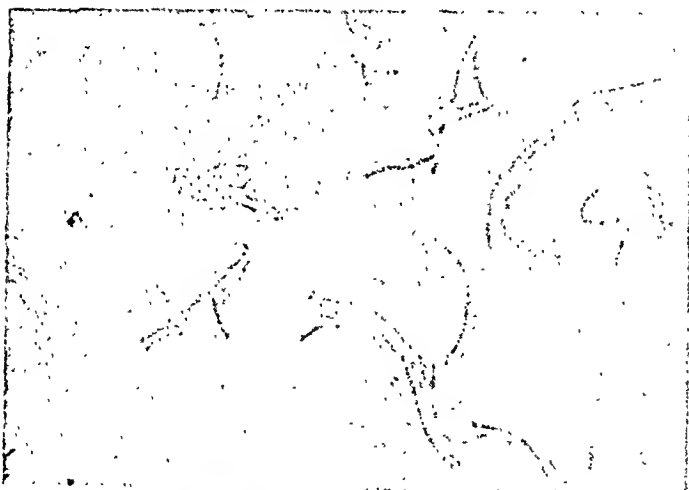


Fig. 2.
2. hrs. 20.00. Magn. 650 \times .

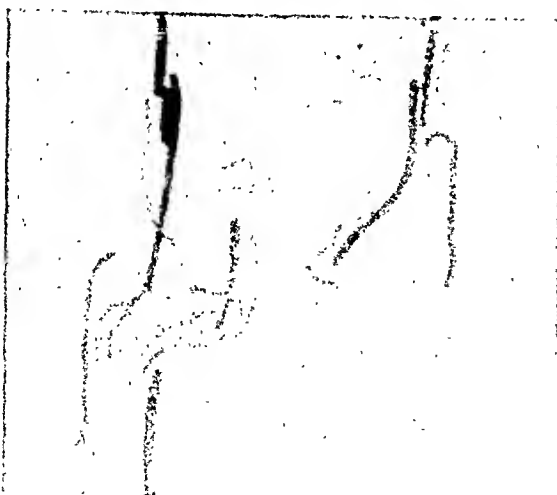


Fig. 3.
3. Carbol-fuchsin stain. Magn. 1450 \times

to classify the swarmers in two types, namely those that will live on and those that disintegrate. But shortly after the swarmers have quietened down, it appears that the individuals are different in their structure. Some are compact and give good contrast to their surroundings and look like wellfilled sausages, whereas others are pallid and give a poor contrast to the surroundings, often lying poorlooking and reminding more or less of empty sausage-skins. At this stage there is no difficulty to see which cells are capable of living.

It may be objected against the investigations so far referred to, that in spite of all, there is a possibility of bio-chemical conditions being the cause, that so many swarmers disintegrate when they, as young individuals, are transplanted to a new milieu. It might be supposed that in the milieu, where the swarmers were hatched, there were stimulating factors necessary for the ripening of the cells into a divisionable condition. Or plainly, the decisive question would be, whether it could be shown that a greater or smaller number of cells decay even in the milieu where they were hatched. This question has already been hinted at several times, the fact having been pointed to, that in impression preparations swarmers were often found, that had all the characteristics of ghost cells.

To clarify this problem, impression preparations were taken with short intervals from a definite part of a swarming zone. It then appeared that in preparations taken at a definite point of time, there were lots of swarmers with indications of degeneration, and in the next preparations the number of ghost cells had decreased, the explanation of which must be, that they had decomposed.

The phenomenon that some of the swarmers, after finished swarming period, do not divide, nor survive, therefore seems to be a physiological phase of the microbes development cycle.

Summary of the finds that have been made.

In part 1 of this study, it has broadly been accounted for the life-cycle which *Proteus* Hauser goes through. On basis of the observations made in this work, it is possible to add some details to the above description of the cycle.

Hauser himself, and most of those who later studied *Proteus* more in detail, have kept their attention on, and emphasized as characteristic for this microbe, that it alternately appears as long and short cell-forms. As far as possible a summary survey of this alternation will be given:

The changing phases with appearance of short and long cell forms go on, as long as the microbe lives in a reasonably favourable milieu. If the conditions of life deteriorate, e. g. by accumulation of toxic metabolites and because of reduced nutritial conditions, this phase-changing will stop. The microbes are then found as short, more or less

fusiform, bodies. Division does not take place, and it might perhaps be appropriate to assume that the cells in this morphological form are in a sort of torpor. When the cells are transferred from such a hunger milieu to a favourable one, they at first seemingly improve their general condition, and then pass to a multiplying phase. After a greater or smaller number of divisions, the multiplying phase ends, and if the conditions of life are optimal, all cells greatly increase their length and develop into swarmer, which in the swarming phase are very mobile and active. After the swarming period it appears that only a smaller number of swarmer are able to divide and thus fit to survive. The majority of the developed swarmer dies and disintegrates. The swarmer capable of living, first pass through a primary division into segments, which in their turn pass into a multiplying phase. In this phase some cells abscind a short piece of one end. As far as it has been possible to observe, these »end-pieces«, have shown no indication of moving, multiplying or developing in any way. It has been observed that a short cell, after it had delivered an »end-piece«, in a short while grew into a swarmer.

Discussion:

In the course of the time this work on the life-cycle of *Proteus* has been going on, and as new details have been clarified, the feeling has increased that the full understanding of the biology and sociology of the microbe becomes increasingly evasive.

One has, however, become more and more convinced that the orthodox view of the bacteria, as very simple beings, which live and multiply by simple bi-partition, is far from the truth. In Part 1 of this work the results agreed with those of many other students, viz. that *Proteus* contains desoxyribonucleic acid, a material which to-day is regarded as a sure indication of a genetic apparatus. The possibility was also mentioned, that the swarmer might derive from »monogranular« »end-pieces«, and that they therefore might be conceived as haploid cells, which became divisionable after having interchanged certain nuclear matter. This conception of the origin of the swarmer appeared not to be correct. But the more detailed studies, of which an account is given in this part of the work, reveal other things which strongly indicate that the swarmer consist of two different types, and also the fact, that some cells deliver a granule before they grow into swarmer, support the same idea.

These facts must inescapably lead to the question whether the swarmer might be regarded as gametes and the whole swarming phase as a sexual phase of the development of the microbes.

Before the discussion of this question, it is necessary to illustrate by some historical data:

From the first period of the bacteriology up to the latter years,

the majority of students has supported the supposition that bacteria represent the lowest step in the phylogenetic development of the animal/vegetable kingdom.

The bacterial cell has in accordance with this view been regarded as an undifferentiated protoplasm-form, without nucleus, which multiplied only by simple bi-partition. A thorough discussion of the position of older bacteriologists to this problem is found in »Handbuch der pathogenen Mikroorganismen by *Kolle, Krauss und Uhlenhuth*, to which it is referred for full details. Here only authorities as *Fischer* and *Migula* shall be mentioned among those who advocate this view.

There were, however, among the older bacteriologists a minority, which represented other interpretations. The controversy about the structure of the bacterium may be brought to mind. Some maintained that the bacterium had a nucleus, and other regarded the microbe as a bare nucleus. Others again, went a step further, and not only maintained that the microbes had nuclei like other cells, but suggested also that the multiplication of the bacterium was far more complex than commonly supposed.

Among those of the last-named group, who go furthest, *Almquist* deserves special attention:

In carefully planned and performed experiments he has shown that several species of bacteria under varying conditions of growth, show certain morphological changes, and that the same bacteria, in a milieu which must be considered favourable, show morphological variations which indicate a far more complex modus of multiplying, than what conforms with the teaching of *Fischer* and *Migula*.

Almquist claims to have shown that the bacterium in a phase of development, which he calls the plasmodial stage, has a diploid nucleus. The plasmodial stage must be supposed to be a rest stage.

Transplanted to favourable conditions of life, there very soon comes a reduction — and the cells multiply further as haploids. This author also maintains that he often has seen cospores, and that he also has observed male cells. (Antheridia).

On this observation, he bases his contention of the sexual reproduction of bacteria — although he admits that he has not observed the copulation itself.

Almquist's terminology — and his analogies with protozoons and fungi — has no doubt partly caused that his works and results have been misunderstood by many. The different morphological forms which this author has placed as natural links in a more complicated life cycle, are by others called involution forms — a conception which certainly often may have been an empty phrase used without explanation or motivation.

It is possible that *Almquist* partly draws too extreme and definite conclusions from his finds, but that he has shown that several bacterial species have a complicated life cycle, can hardly be doubted.

From newer observations in the same field, references are given to the works of the following authors:

Allen, L. A., Appleby, I. C., and Wolf, I.

These authors have experimented on various species of sporates through their life-cycle, »From extreme youth to old age«, and shown the cytological development step by step. They have showed that during the multiplying stage there appear granulae in the cells, and that these cells liberate these granulae, and that it may be possible that they are of vital importance. »They were seen outside the cells, giving the impression that they are liberated and may have some vital function«. The authors claim that in their life-cycle, cells of both haploid and diploid nature occur, the haploids during the multiplying phase.

Abnormal cells, clubs, balls or cells with buds etc.) they claim to have seen often. »Their origin is uncertain, but probably they arise in cells which have failed to sporulate, from the effort to adapt themselves to the unfavourable environment of the ageing culture«.

In later years the morphology and genetics have been the object of intense studies, and so many data are so well clarified, that many of the problems formerly under discussion, can be explained out from general biological considerations.

Modern methods of examination have shown that the bacteria contain the same material, that in other cells is regarded as a specific part of the genetic apparatus, or in other words it must be supposed that the bacteria like other cells have nuclei.

On the basis of such a revision of the cytology — or more carefully put, the composition, of the bacterial cells, it is possible to explain that definite characteristics of bacterial species re-appear from generation to generation — a problem that before was rather incomprehensible.

Parallel with the exploration of the structure of the bacterial cell, experimental results have been obtained in later years, which may indicate that several bacteria have sexual reproduction, and that this process not necessarily must take place by copulation of cells — but may happen by indirect transfer of specific nucleus substance.

Griffith is no doubt the first who by general acknowledgement has shown that the characteristics of one bacterial cell can be transferred to another. His transformations of capsule-free, non-pathogenic pneumococci to capsulated pathogenic ones, may seem to involve the same principle as cross-fertilization, even if one party to the act, capsule-free cell, was killed by heat. The detailed mechanism of *Griffith's* transformation, is explained by *Avery* and his collaborators, who were able to show that the active substance was desoxyribonucleic acid, which also was effective in a chemically purified form.

Further proof of sexual propagation among bacteria was given by *Tatum* and *Lederberg*. These workers mixed two bacterial strains of

different bio-chemical characteristics, and isolated types which might be explained as bastards.

The conclusions of these new discoveries within the cytologi of the bacterium, must be, that it is difficult to deny that hereditary characteristics can be transferred from one bacterial cell to another, and that this process does not imply copulation of cells, but that an interchange of filterable nucleal substance is sufficient.

With these facts as a support, the discussion of the question whether the *Proteus* swarmers can be taken as gametes, will be continued.

The most common interpretation of the swarmers has been that they are morphologic degeneration forms which develop in an inferior milieu, and that they have no specific biologic function. This interpretation has been refuted in a previous paper, and it has been shown that the swarmers are dependent on optimal conditions of life. *Russ-Münzer's* claim, that swarmers develop only on solid media and that their »purpose« is to bring the culture to new »pastures«, cannot be upheld against critic. It is certainly unquestionable that the swarmers also develop in fluid media. None of the explanations of the development and biological rôle of the swarmers which has been possible to trace, is satisfying.

As the later studies of the development of the swarmers show, their rôle cannot be that of aiding the multiplication of the species — on the contrary, the fact that the greater part of the swarmers dies, shows that the swarmer phase actually involves a strong reduction of the bacterial population. In this connection it should be borne in mind, the fact well known from the protozoology, that a sexual phase in a cycle of development, often means a reduction of a population, e. g. hologamy, which reduces the number of individuals by a half.

Hologamy is a copulation, and it has been objected against those who have assumed sexual reproduction in bacteria that copulation has never been observed with certainty.

But against this objection it can be pointed to the many and complicated modi in which fertilization takes place by conjugation among protozoa. That conjugation *can* take place, is hardly possible to deny, and that this fertilization modus in *principle* can be supposed to take place, is evident from Griffith's and Avery's results.

The pattern of division has been accounted for before. The mitotic division of the intra-cellular, regularly arranged granules must lead to the thought of a reduction and create a suspicion that in the cycle there are both diploid and haploid cell forms, which again must mean that somewhere in the cycle there is a sexual phase.

In the same direction it is possible to explain the phenomena that has been observed, that some cells in their multiplying phase abscind an »end-piece« while developing into swarmers. Up to now, to be

neutral, these abscinded pieces have been called only »end-pieces«. But it is possible that here is the development of a macro-gamete and that the »end-pieces« are polar bodies.

So many details point to the possibility that the swarmer phase is a gamete phase, that it must be permitted to give some suggestions as to how the cycle, based on this supposition, may be perceived.

As is well known from the protozoa, both the conjugants may be quite identical in form and biology. They are fertilized bilaterally, and both parties are divisionable and live on.

This explanation of the swarming phase raises the question of how the great mortality among the swarmers can be fitted in.

It might be supposed that a greater number of individuals for some reason become incapable of fertilization and therefore dies.

Another explanation, which seems to fit the observations better, is that the conjugants are differentiated macro- and micro-gametes.

By such hypothesis it is possible to give a natural explanation of the »end-pieces« as polar bodies. The great mortality among the swarmers will then get a plausible explanation — as it is well known that micro-gametes often are far more numerous than macro-gametes.

The swarmers capable of living *must then be supposed to be zygotes*, and the nuclear relations of zygotes presumably must be such that several diploid nuclei are formed in the same individual, discernible as the regularly arranged granules — and that a reduction takes place, which explains the bi-partition of the granula.

The partitioned segments then get each two haploid nuclei and the »end-pieces« each one haploid nucleus.

Summary.

1. The development of the swarmers in *Proteus* was studied.
2. Evidence is presented that the short actively multiplying cell from in favourable conditions differentiates into swarmers after a number of mitoses.
3. It is suggested that the change from the multiplying cell phase to the swarming phase is induced by some biochemical process in the growing colony.
4. Only a small fraction of the multiplying cells »deliver« a granulum from the one end before they grow into a swarmer.
5. Studies of swarmers which were transferred to a new medium at different stages of development have shown that only a small fraction of the individuals develop into cells, capable of division and continual life, whereas the swarming period seems to be the terminal stage of the majority of the individuals.
6. Considerable evidence indicates that the swarmers may be sexual cell forms and represent a definite stage in the life cycle of the organisms.

It is conceivable that swarmers capable of division may be zygotes, containing diploid nuclei at the initial stage. The division of the swarmers through the middle of the regularly arranged granules may then represent a reduction, and the short cell forms accordingly might be regarded as haploid in constitution, with one haploid nucleus at each end.

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ON THE CLASSIFICATION OF THE ENTEROBACTERIACEAE*)

By F. Kauffmann.

(Received for publication on June 9th, 1949.)

By the term Enterobacteriaceae we mean a large family of Gram-negative, non-sporing rods, either motile with peritrichous flagella or non-motile. They grow on ordinary media and ferment glucose rapidly with or without gas production. They reduce nitrates to nitrites. This family can be divided into biochemically defined groups in the following way:

Enterobacteriaceae

Groups	Tribes
1 Salmonella	Salmonelleae
2 Arizona	
3 Bethesda	
4 Ballerup	
5 Escherichia	Eschericheae
6 Alkalescens-Dispar	
7 Klebsiella	
8 Serratia	
9 Shigella	Shigelleae
10 Proteus	Proteae
.	
.	

*) Abstract of a lecture given in the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia, U. S. A. on May 16th, 1949.

The principle of our classification is to establish biochemical groups which are divided into serotypes. There is no single character which is exclusively characteristic of a certain biochemical group, but as a rule a combination of some characters will give a diagnosis.

There are no sharply defined groups in nature, their delimitation is more or less arbitrary. In contrast hereto, we are able clearly to define the individual serotypes which are the fundamental units. The one important point is therefore the serological analysis of the individual types.

I should like to omit the terms »tribes«, »genera« and »species«, but if it is wanted to combine several genera (= groups) into tribes, I suggest that the groups *Salmonella*, *Arizona*, *Bethesda*, and *Ballerup* be entered together in the tribe »*Salmonelleae*« as these groups are closely related biochemically as well as serologically. In that way we avoid a special tribe »*paracolobactrum*« (1) or »*paracolon*«. In contrast to Bergey's Manual (2) I would not refer the *Shigella* group to the tribe *Salmonelleae*, but rather suggest a special tribe *Shigelleae*. I presume that genetically the *Shigella* group stands on the opposite side of the *Escherichia* group and that the development has gone in two opposite directions: one towards the *Salmonella* group, the other towards the *Shigella* group. But this is only a hypothesis, as the genetic relationships of *Enterobacteriaceae* are quite unknown.

With regard to the tribe »*Eschericheae*« (according to Bergey's Manual) I propose that the *Escherichia*, *Klebsiella*, and *Serratia* groups be entered together into one tribe. The »*Aerobacter*« group is combined with the *Klebsiella* group (3). Related to this group is the *Serratia* group which is characterized by its pigment formation (*Prodigiosus* group). Orienting serological studies carried out with a few strains showed that two strains contained the O antigen 19a found in the *Escherichia* group.

The *Alkalescens-Dispar* strains, which previously have been reckoned to the *Shigella* group, belong biochemically and serologically to the *Escherichia* group: they are non-motile, often anaerogenic and lactose-negative *Escherichia* strains. Serologically they belong to the same frequent O groups (1, 2, 4, 9, and 25) as typical *Escherichia* strains. They contain such K antigens as also often occur in *Escherichia* strains, e. g. the L 1 antigen. Further details will be reported on later by my co-worker Frantzen.

Alkalescens strains are 2 of H. Braun's »*Flexner*« strains, designated as »*Clark*« and »*Üstün*« (4). They belong to *Escherichia* O group 25 and contain the L antigen 1 of the *Escherichia* group. Biochemically they behave like *Alkalescens* strains, i. e. they differ from true *Flexner* strains.

Recently a *Shigella* Commission within the International Association of Microbiologists — consisting of Boyd (as chairman), Bridges, Carlquist, Ewing, Ferguson, and myself — has been working on

the classification and nomenclature of the *Shigella* group and a report on this subject will be given during the next International Congress.

Turning more to the *Proteus* group, I can refer to a paper of my co-worker Beate Perch (5) who lately has published a diagnostic antigenic schema for the *Proteus* group (*Proteus vulgaris* and *mirabilis*).

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COMPLEMENT FIXATION EXPERIMENTS IN CORYNEBACTERIUM DIPHTHERIAE

By *Per Oeding.*

(Received for publication June 22nd, 1949.)

The complement fixation test has rarely been used in serological examinations of the diphtheria bacillus. *Kolmer* (1912) and *Przewoski* (1912) could not distinguish between different strains of *C. diphtheriae* by means of complement fixation, and *Mansheim* (1930) did not even get fixation with the homologous strain. In 1933 *Menton*, *Cooper* and *Fussell* published the results of complement fixation examinations on 200 diphtheriae strains. The types *gravis*, *mitis* and *intermedius* were found to be serologically closely related, particularly *gravis* and *intermedius*. In many instances, however, there was no correlation between the cultural type and the complement fixation test, which also diverged considerably from the later classification of the diphtheria bacillus by means of agglutination, initiated by *Orr Ewing* (1933).

As antigen in their complement fixation tests *Menton*, *Cooper* and *Fussell* used formalin-killed suspensions in saline of 24 hours' old agar cultures. No fixation was observed when dilutions of serum were made, and they therefore used a constant serum dose and a dilution of the antigen, starting with as concentrated antigens as 10 times the concentration used in complement fixation with *Salmonella*.

Experimental.

It might in our opinion be of interest to see if it should be possible to find a more convenient method for the demonstration of complement fixing antibodies. The results of *Menton*, *Cooper* and *Fussell* were in bad accordance with the cultural and agglutinative classification of the diphtheria types, and it seemed probable that the divergence was caused by the technique which they used in their complement fixation tests.

Methods.

Different media and methods were examined with the intention of producing a good antigen for the complement fixation test. Antigens made from Loeffler cultures proved to be strongly anti-complementary, both when they were suspended in saline and after freezing and thawing 10 times. As a result of the anti-complementary activity the antigens were very dilute and gave no definite complement fixation with homologous serum. The best antigen was obtained when the growth on 7% human blood agar plates was suspended in saline and heated in water bath at 56° for 1 hour. This antigen showed no anti-complementary activity even in high concentrations.

The strongest complement fixation was seen in antigens containing about 2000 millions bacteria per ml. More concentrated antigens could not be used, owing to the turbidity at higher concentrations. With less concentrated antigens than 2000 millions per ml the specific fixation quickly decreased (table 1).

Table 1.

The significance of the concentration of the antigen in complement fixation tests.

	Serum dilution			
	48	96	192	384
<i>Antigen gravis str. 8.</i>	<i>Serum gravis str. 8.</i>			
2000 mill. bact./ml.	+	+	+	0
1000 - - -	+	+	+	0
500 - - -	+	+	0	0
250 - - -	0	0	0	0
<i>Antigen mitis str. 24.</i>	<i>Serum mitis str. 24.</i>			
2000 mill. bact./ml.	+	+	0	0
1000 - - -	+	0	0	0
500 - - -	0	0	0	0
250 - - -	0	0	0	0

Controls: 0

++ + + no hemolysis

++ +, + +, + increasing hemolysis

0 complete hemolysis.

Antisera were produced by injections on rabbits of formalin-killed Loeffler cultures, after the method used by *Tarnowski* (1932). Agglutinating titres varying from 10240 to 320 were obtained. Gravis strains ordinarily gave more potent sera than mitis and intermedius strains. As some of the antisera might be slightly anti-complementary, dilutions of the sera in saline 1/4 were used. The first dilution in the complement fixation test thus was 1/48. Each tube contained 0.2 ml of each reagent, altogether 1.0 ml. The complement dose was 2 hemolytic units, and the amboceptor dose 4 hemolytic units. 3% washed sheep blood corpuscles were used. Fixation took place in a water bath at 37° for 1 hour, when blood and amboceptor were added, and the results read after 10 minutes at 37°. When the complement fixation was performed in the refrigerator over night the results were not as good as after 1 hour at 37°. Serum and antigen controls were set up in each test.

Results.

Complement fixation tests were performed with a series of freshly isolated gravis, mitis and intermedius strains, which had been thoroughly examined and typed culturally, and serologically by means of agglutination. Table 2 demonstrates cross complement fixation tests between 12 strains, and the titres may be regarded as representative.

The results of the complement fixation tests in many respects differ from those obtained by agglutination. Firstly it is striking that the titres are considerably lower, only 384 in the gravis strains, which agglutinated to a titer of 10240 in the homologous serum, and even lower in mitis and intermedius. In addition considerably stronger cross reactions were seen between the types gravis, mitis and intermedius than in the agglutination tests, where only quite insignificant cross reactions were observed. As we have used highly potent antisera and good antigens, the low homologous titres and the strong cross reactions in complement fixation tests as compared with agglutination tests are difficult to explain. It is possible that the fixation of the complement to the antigen-antibody complex somehow is incomplete, or that other antigens, chiefly group-antigens, take part in the reaction in the complement fixation test. The complement fixation experiments indicate a nearer antigenic relationship between gravis and intermedius than between gravis and mitis and mitis and intermedius.

Table 2
Cross complement fixation tests between 12 diphtheria strains

Antigen		Antisera											
		Gravis					Mitis					Intermed.	
		1	8	357	364	369	24	76	122	397	4037	3764	499
Gravis str.	1	384	384	384	384	192	48	0	0	0	0	96	96
-	8	384	384	384	192	192	48	0	0	0	0	96	96
-	357	384	384	384	384	192	48	0	0	0	0	96	96
-	364	384	384	384	192	192	48	48	0	0	48	96	96
-	369	384	384	384	384	384	96	96	0	0	0	96	96
Mitis str.	24	0	48	48	48	48	96	96	48	0	48	0	0
-	76	0	48	48	0	96	96	96	0	0	0	0	0
-	122	48	96	96	48	48	96	48	96	48	96	48	48
-	397	0	48	48	48	48	48	0	48	192	48	48	48
-	4037	48	48	48	48	48	48	0	96	48	96	0	0
Interm. str.	3764	96	96	96	96	96	0	0	0	0	0	96	192
-	499	96	96	96	96	96	0	0	0	0	0	96	192

Controls: 0.

The 5 gravis strains all belonged to Robinson and Peeney's type II. Mitis strains 24 and 76 belonged to the same agglutinative type, whereas the other mitis and the 2 intermedius strains represented different agglutinative types.

Discussion.

In spite of using highly potent agglutinative antisera and good antigens only small amounts of complement fixing antibodies could be demonstrated. As also considerable cross reactions were seen in complement fixation tests, it was evident that other antigen-antibody reactions, probably mainly group reactions, take place in complement fixation tests than in agglutination tests. So far our findings are in accordance with the results of *Menton, Cooper* and *Fussell*. These authors, however, had to make dilutions of the antigen, as no fixation was observed with the usual technique with serum-dilutions, and by this method got a number of discrepencies between cultural and serological classification.

Although the titres in our experiments are low, a specific fixation was demonstrated which was in fairly good accordance with the results obtained in the agglutination tests. The complement fixation test is, however, not suited for the serological classification of the diphtheria bacillus in types.

Conclusions.

1. Good antigen of the diphtheria bacillus for complement fixation tests was obtained on blood agar plates.
2. The titres were considerably lower and cross reactions stronger between gravis, mitis and intermedius than in agglutination tests.
3. The complement fixation test is not suited for the serological classification of the diphtheria bacillus.

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ON THE NATURE OF THE METACHROMATIC GRANULES OF THE DIPHTHERIA BACILLUS

By *Per Oeding*.

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The granules of the diphtheria bacillus were by *Ernst* (1888) originally interpreted as spores. *Neisser* (1888) observed that they were acid-fast and *Babes* (1889) that they got a reddish colour with methylene blue (metachromatic granules). *Grimme* (1902) maintained that the granules consisted of reserve food and called them volutin granules. This view was supported by *Meyer* (1904) and has later been generally accepted. *Grimme* and *Meyer* indicated the chief methods by which volutin could be distinguished from other cell inclusions and protoplasmatic structures. The volutin granules are acid-fast, are stained more vigorously with basic aniline dyes than the remaining protoplasm and are dissolved by heating and by alkalies and certain acids.

Pesch (1924) stated that organic or inorganic phosphorous compounds were necessary for the development of the granules, and *Schumacher* (1922) that they consisted of free nucleic acid, as they were coloured green with methylene blue and chrysaniline.

Some authors were of the opinion that the metachromatic granules had something to do with the propagation of the diphtheria bacillus. Thus *Marx & Woithe* (1900) assumed that the granules took part in the division of the cells and *Gróh* (1938) that they were living individuals which burst out of the mother cell and grew up to new individuals. This must be seen in relation to *Hadley, Delves & Klímek's* (1931) description of a filtrable phase of the diphtheria bacillus.

The composition and function of the metachromatic granules have acquired new interest after the description of 3 types of the diphtheria bacillus by *Anderson et al.* (1931). *Mitis* was characterized by many and big granules on ox serum, whereas *gravis* had few and small

granules. Mair (1934) and Murray (1935) found that intermedius was characteristically barred.

Structures in the protoplasm of bacteria reminding of nuclei have been demonstrated by means of Feulgen's reaction [Feulgen & Rossenbeck (1924)] by various authors during the last years [Boivin & Vendrely (1947)]. With Feulgen's reaction the ribonucleic acid in the protoplasm is dissolved and the remaining thymonucleic acid or desoxyribonucleic acid takes a violet colour. Desoxyribonucleic acid is known as a component of nuclear material. Other authors have, however, regarded the reaction as non-specific, and diffusely distributed colour or coloured granules as reserve food and not real nuclear material.

Experimental.

It was our intention to study the metachromatic granules of the diphtheria bacillus after heating and with Feulgen's reaction, to see if they should be regarded as volutin or as nuclear material. It would also be of interest to see if there could be demonstrated any difference in this respect between gravis, mitis and intermedius.

Various strains of gravis, mitis and intermedius were suspended in distilled water after 18—24 hour's growth on Loeffler slants. A Neisser smear was made of each strain, whereas the remaining suspension was heated for 1 minute in water bath at 100° and then stained in the same way. The smears of each strain were then compared before and after heating.

It was demonstrated that strains which before heating had many big metachromatic granules lacked stainable substance afterwards. There was no difference in this respect between the 3 types. After heating the diphtheria bacillus got a considerably more barred, unevenly stained appearance than before heating. This was also observed in strains which had only few metachromatic granules before heating, and the punctured appearance is therefore not here due to disintegration of the granules. The explanation may be that volutin which does not stain with Neisser's stain is distributed in the bacteria, and that the dissolution of these granules causes the punctured appearance. When one of the 3 types of the diphtheria bacillus, mitis, is distinguished by much bigger and more metachromatic granules than the other types, the explanation of this might rather be a different chemical composition and staining of the volutin of the 3 types than a quantitative difference.

With Feulgen's reaction no localized nuclear substance was demonstrated, neither corresponding to the metachromatic granules, nor in the remaining part of the bacteria. The same results were obtained in cultures of different age, which were examined after 6 hour's growth on Loeffler slants and daily for several days, and in up to 18 months old cultures in broth. There was no difference between gravis,

mitis and intermedius in this respect. In all examinations, however, the individuals had exactly the same appearance with Feulgen's reaction, i. e. a uniform, light violet colour of the bacteria. Controls with different solutions gave the same positive Feulgen's reaction.

Discussion.

Our experiments have confirmed earlier investigations on the nature of the metachromatic granules of the diphtheria bacillus. If, as *Grimme* states, heating of the bacteria is a reliable method in distinguishing volutin from other cell inclusions, it must be assumed that the metachromatic granules of the diphtheria bacillus consist of volutin. In good accordance with this no nuclear material was demonstrated in the metachromatic granules by us, when Feulgen's reaction was used. The diphtheria bacillus, irrespective of the growth curve or the type of bacteria, however, always got a light violet colour. If a positive Feulgen's reaction gives a reliable expression of the presence of nuclear material, our results should indicate that the diphtheria bacillus has got no localized nucleus, but nuclear material diffusely distributed through the cell. Similar results have recently been demonstrated by *Kvittingen* (1949) in *Proteus* in its swarming phase.

Conclusions.

1. The metachromatic granules of the diphtheria bacillus consist of volutin.
2. With Feulgen's reaction no nuclear material was demonstrated in the metachromatic granules. The bacteria got a light violet colour, indicating the presence of diffusely distributed nuclear material.
3. These results were obtained irrespective of bacterial type or age of culture.

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THE FERMENTATION OF DEXTRIN AS AN AID IN THE TYPE DIFFERENTIATION OF THE DIPHThERIA BACILLUS

By *Per Oeding*.

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In their description of 3 types of the diphtheria bacillus, *Corynebacterium diphtheriae* gravis, mitis and intermedius, *Anderson et al.* (1931) stated that gravis fermented starch and glycogen whereas mitis and intermedius were inactive. Gravis also fermented dextrin but not d-glucosamin, whereas mitis was inconstant against dextrin but fermented d-glucosamin. They assumed that mitis did not actually ferment dextrin, and that the inconstant acid formation was caused by impure preparations.

The fermentation of d-glucosamin was found to be inconstant by later authors, and d-glucosamin did not therefore attain any significance in the biochemical type-differentiation. Nor did the fermentation of dextrin get any practical significance, as later authors did not find any difference in the fermentation of dextrin by the 3 types, and some authors maintained that the fermentation of dextrin was unreliable as the dextrin preparations unfrequently were pure [*Barratt* (1924), *Okell & Baxter* (1924), *Frobisher* (1938), *Tarnowski* (1942)].

Starch or glycogen are now the only sugars which are used in the differentiation between the 3 types of the diphtheria bacillus. Many sugars have been examined but no one was differently fermented by the 3 types [*Frobisher* (1938), *Tarnowski* (1942)]. As some authors did not get reliable results with starch, a sugar reaction beside starch might be of value in the type differentiation, and especially if it could distinguish between mitis and intermedius, none of which are fermented by starch.

Experimental.

In an earlier paper (1949) we have emphasized the importance of using a simple medium such as peptone-water in the sugar reactions of the diphtheria bacillus. All the diphtheria strains examined grew

well in peptone-water, with strong reactions after 1 day. False starch reactions, which have frequently been reported when the fermentation reactions have been made in broth or serum-water, are thereby avoided.

As we noticed that some diphtheria strains gave strong whereas others gave weak reactions in galactose, glycerol and dextrin, further examinations were made with the intention to see if there should be any difference between *gravis*, *mitis* and *intermedius* in this respect.

Table 1.

The fermentation of dextrin by 105 diphtheria strains.

Type	Dextrin	
	Strong reaction	Weak reaction
Mitis	2	37
Gravis	58	6*)
Intermedius	2	0

The strains were typed culturally, biochemically and serologically.

*) 2 of these strains were culturally atypical, weak starch fermenters and belonged to our serological *gravis* type F 4680 (*Tarnowski's gravis* type 1023). 61 of the *gravis* strains belonged to *Robinson & Peeney's* type II.

Table 2.

The differentiation between the 3 types by means of starch and dextrin fermentation.

	Starch	Dextrin
Gravis	+	strong reaction
Mitis	÷	weak reaction
Intermedius	÷	strong reaction

The sugar reactions were performed in 1 % peptone-water (Parke Davis & Co.) with Andrade's indicator and 1 % sugar (pH 7.8). With the exception of starch all sugar solutions were heated to 100° C. in an Erlenmeyer flask, distributed in 10 mm. wide tubes and finally placed in streaming vapour for ½ hour. The tubes were inoculated with 2 drops of a 24 hours old phosphate broth culture. They were incubated in vertical position at 37° and controlled daily for 14 days. 2 controls were used: (1) uninoculated sugar tubes, (2) cultures in peptone-water with indicator but without sugar.

All the 105 diphtheria strains examined fermented galactose and glycerol, but some strains fermented these sugars weakly, particularly glycerol. There was, however, no connection between the reactions in

these sugars and the cultural type, whereas the types differed clearly in their fermentative power against dextrin (table 1). With few exceptions *gravis* gave strong acid formation with dextrin after 1 day, and *mitis* weak. As *intermedius* is found very rarely in Norway, only 2 strains of this type were examined; both fermented dextrin strongly as *gravis*. The results indicate that *gravis*, *mitis* and *intermedius* may be distinguished by means of their fermentation of starch and dextrin, whereas starch alone will not separate *mitis* from *intermedius* (table 2).

In our experiments only one dextrin preparation (»Merek«) was used, as other preparations were not available. When the sugar reactions are made in peptone-water with 5 % inactivated serum, instead of in peptone-water only, the difference between the types disappeared, and even the *mitis* strains ordinarily gave strong reactions with dextrin.

Discussion.

In the fermentative separation between the 3 types of the diphtheria bacillus only starch or glycogen have till now been used. No »sugar« has been found which could distinguish *mitis* from *intermedius*. In our experiments dextrin was found to be a valuable aid to the type distinction of the diphtheria bacillus, when the tests were made in peptone-water. Dextrin should therefore be used as a routine beside starch or glycogen. As *gravis* gives strong acid formation with dextrin and *mitis* weak, dextrin will support starch in the distinction between these two types. Our 2 *intermedius* strains gave strong reactions with dextrin as *gravis*, and if this proves to be constant in the intermediate type, it will be possible by means of starch and dextrin to separate the 3 types from each other.

Anderson *et al.* (1931) assumed that *mitis* did not actually ferment dextrin, and it is possible that the weak reaction of *mitis* is due to the presence of small quantities of glucose in the preparations. It is essential that a simple medium such as peptone-water is used in the sugar reactions, as no difference was seen between the reactions of the 3 types in dextrin when 5 % serum-peptone-water was used.

Conclusions.

1. *Gravis* and *intermedius* (2 strains) give strong acid formation with dextrin in peptone-water, whereas *mitis* gives weak acid formation.
2. Dextrin supports starch in the differentiation between *gravis* and *mitis*, and may separate *mitis* from *intermedius*.

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STUDIES IN MUCOID ESCHERICHIA COLI I. DISSOCIATION AND MUTATIVE FERMENTATION

By *Sverre Dick Henriksen.*

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In the course of a study of some mucoid strains of *Escherichia coli* a few of the strains were found to be unusually variable with respect to colony type and lactose fermentation. Most of the variations conformed with usual patterns, but in some cases they seemed to take an unusual course. Some of these observations seemed worth recording.

The results of a serological study of the strains are presented in the following paper. In this paper observations relating to colony dissociation and to mutative lactose fermentation will be described, and only serological data as are pertinent to the study will be used.

Origin of the strains.

Case 248/48. Chronic cystopyelitis. The primary bromthymol blue lactose agar plate culture contained two different colonies: 1. Large, domed strongly mucoid, translucent colonies with the consistency of thin agar jelly (248 M). 2. Ordinary coli colonies, smooth, moderately opaque, low convex and creamy (248 S). The strains had the same O-antigen and were considered as different mutants of the same strain.

Case 700/48. Chronic cystopyelitis. The primary culture contained 3 different colonies: 1. Large, domed, strongly mucoid, semitransparent blue colonies of a similar consistency as 248 M, but slightly stringy. (700iML—). 2. Flat, smooth or slightly granular, blue colonies of a somewhat dry consistency. They were unstable in saline, strongly anti-complementary in complement fixation tests, but otherwise had the same properties as 700iML—. They were believed to be R-forms of this strain (700iRL—). 3. Colonies of about the usual size, moderately mucoid, glistening and confluent, yellow, often with an opaque centre and a more translucent periphery. The consistency was soft creamy. The strain differed from the two former in saccharose fermentation and in the O-antigen and was considered to be a different strain (700em).

Case 411/48. Chronic cystitis. The colonies on bromthymol blue lactose agar

were very similar to those of 700₁M, but the colour tended to be more greenish (411 ML—).

Case 567/48. Chronic urinary infection. The colonies were large, domed, occasionally with a slight depression of the top, strongly mucoid, slightly translucent, yellow and of an extremely tough, viscous consistency. (Strain 567 M). Strain 173 M was the same strain isolated from the same patient several months later.

Case 41/45. Ureter fistula after lithotomy. The strain was mucoid when first isolated, but very soon lost its capsules and became S. After storage on agar tubes for about two years with monthly transfers, however, it suddenly reverted partially to the M-phase. The mucoid (41 M) and smooth (41 S) strains were very similar to 248 M and S.

Case 60/48. Chronic urinary infection. Two strains, exactly similar to 248 M and 248 S respectively, were isolated (60 M and 60 S).

Case 329/48. Chronic urinary infection. The colonies at first grew very slowly, but gradually gained a higher growth energy. They were large, strongly mucoid, translucent, of a very viscous consistency although not quite as tough as 567 M. The colour on bromthymol blue lactose agar was light green, gradually turning yellow (329 MG).

Case BJ. Secondary infection of ulcerating sarcoma. 2 strains were isolated: 1. Fairly large, domed, strongly mucoid colonies, resembling those of 248 M, but slightly smaller (Bj₁M). 2. Small mucoid colonies resembling those of 700₂m (Bj₂m). The strains differed from each other in fermentation and in motility, the latter one being motile. They were considered as different strains.

Biochemical reactions.

These were typical of *E. coli*, with a few minor deviations. Thus the strains 248 M and 248 S failed to produce gas from glucose when first isolated, but they very rapidly regained this faculty. 329 MG showed an apparently permanent loss of the faculty of gas production. Strains 700₁ML— and 700₁RL—, although blue on plates, fermented lactose within 24 hours, but showed a very slight delay as compared with the »yellow« strains. Strain 411 ML— did not ferment lactose within 10 days.

Methods.

Two methods were used in the study of bacterial variation: 1. Cultivation on bromthymol blue lactose agar plates at room temperature with transfers only when the plates started to get dry (every 2—3 weeks). 2. Examination of old broth cultures after spreading on the same kind of plates.

As the mucoid strains contained the same M-antigens (see the following paper), tests for the presence of this antigen were made by slide agglutination in an immune serum against an M-strain, which had been exhausted of all somatic antibodies by absorption with the homologous S-strain.

Results.

In order to avoid unnecessary repetition, the variations observed in the individual strain or in a group of strains are described separately.

Strains 248M, 41M, 60M, Bj₁M, Bj₂m and 700₂m.

The S-forms of these strains could be isolated easily from broth cultures of the M-form after one or more days. S-colonies also fre-

quently appeared in subcultures on plates of the M-form. Apparently the S-strains were O-forms as they did not seem to contain any K-antigen and behaved as O-forms in agglutination tests. They all looked like ordinary coli colonies. In two cases two slightly different S-strains were isolated. One of each pair (248S₁ and Bj₁S₁) had a pale yellow color, sometimes with a slightly darker centre, the other (248S₂ and Bj₁S₂) had a darker orange color and often the periphery appeared to have the darkest color. On plain agar plates it seemed possible, once the difference had been recognized, to detect a very slight difference in the opacity of the two types, the pale yellow variety being a trifle more opaque, but the difference would not have been noticed unless attention had been focused on it. There was found no serological difference between the two varieties. Tests for M-antigen showed that this was present in all the M-forms but absent from all the S-forms.

Strain 329MG.

This strain split off S-forms less readily than the others, but they were isolated from a 25 days old broth culture. They were somewhat irregular, resembling R-forms, but in all other respects they behaved as S-forms (329S). On lactose agar plates the colonies of 329MG were slightly smaller than most of the other M-strains, and it appeared as if lactose fermentation might proceed more slowly, as shown by the initial green color, but frequently there appeared a few larger, strongly yellow, less translucent colonies, which, in subculture, mainly reproduced the same type of colony. Most subcultures of this variety (329MY), however, contained a few smaller, paler, more translucent colonies, apparently of the original type. It was impossible to obtain any of two types in a stable form.

Strain 411ML — and 700₁ML —

These strains readily split off S-forms of the same type as in the other strains, but blue on lactose agar (strains 411SL — and 700₁SL —). All these 4 strains were mutable strains and produced yellow secondary colonies, which, on subculture, reproduced stable lactose fermenters. (411ML+, 411SL+, 700₁ML+ and 700₁SL+). The mutation rates, however, were very different. Whereas cultures of 700₁ML — and 700₁SL — always produced several yellow daughter colonies, in the two other strains this occurred only very rarely. Thus the mutation rate of the M and S form of each strain seemed to be approximately the same. This was also shown by the results of fermentation tests in tubes with lactose peptone water. In such tubes the 700₁ strains showed only a very slight delay of fermentation during the first 24 hours, whereas the 411 strains usually remained negative for 10 days or more.

The strains 411ML + and 700₁ML + showed a similar variation as strain 329M, as two types of colonies frequently appeared in the cul-

tures, one larger, more intensely yellow and one smaller, pale and more translucent. These variants could not be obtained in a stable form. The strain 411SL + after some months split off two different colonies, one (411SL +₁) pale yellow and the other (411SL +₂) dark yellow. Apart from these differences the two strains seemed to be identical.

Finally both the strains 700₁SL— and 700₁SL+ produced typical S-colonies on lactose agar plates, but the latter, after a few days at room temperature, produced a slime wall around the colonies. Tests with anti-M serum indicated that this slime wall contained the M-antigen.

The strains 700₁ML — and 700₁SL — produced deep blue colonies on bromthymol blue lactose agar, and remained blue, apart from the production of daughter colonies. The corresponding 411 strains, on the other hand, were bluish green and gradually tended to become more light green to yellowish in the course of a few days. Thus there seemed to be a difference in the behaviour of the two strains towards lactose. One pair of strains seemed to be entirely inactive, whereas the other appeared to cause a slow, gradual fermentation.

Strains 567M and 173M.

These strains showed a very similar behaviour and only the changes observed in the former are described. After a few days in broth, colonies appeared which were slightly smaller and slightly less tough than the original ones (strain 567Mm), but the difference was small. At about the same time still another type of colony appeared. This was much smaller and more opaque, but still distinctly mucoid as shown by the glistening surface and the tendency to form amoeboid colonies (strain 567 m) (fig. 1 and 2). This seemed to be the last step before the appearance of S-colonies. Such colonies were isolated from 9 to 62 days old broth cultures, and they were of several, slightly different types: 1. Typical smooth colonies, but of a stringy consistency (strain 567S(m)). 2. Similar colonies but of a butyrous consistency (567S). 3. Colonies which had a rather dull surface and which were of a more dry consistency, and unstable in saline (567R). All these three types were yellow on the plates but there also appeared some colonies that behaved differently: 4. Smooth but dull, slightly granular colonies which first were yellow but in the course of a few days at room temperature turned the plates blue (567RL + —) 5. Small translucent blue colonies, which also appeared to be rough (567RL —). These latter colonies showed a particularly interesting behaviour as they were very unstable and produced a large number of daughter colonies of different types. Thus this was a mutable strain. By subculture of different daughter colonies it was possible to isolate additional strains. Some of these were the same as the numbers 3 and 4 above, but some were different, namely: 6. Small yellow mucoid

colonies of about the same size and appearance as the strain 567m, but the consistency was distinctly less viscous, only very slightly stringy (567m₂). 7. Small blue mucoid colonies of a similar appearance and consistency as 567m₂. This was a mutable strain and produced yellow secondary colonies, which gave similar colonies as 567m₂ in subculture (strain 567m₂L—). 8. Small fairly smooth, colonies with a dull surface. These after a few days turned from yellow to blue and they also became surrounded by a slime wall (strain 567R(m)L + —).

Tests with absorbed anti-M serum showed that all the mucoid



Fig. 1.

Strain 567M, 7 days at room temperature on bromthymol blue lactose agar. 5 X.

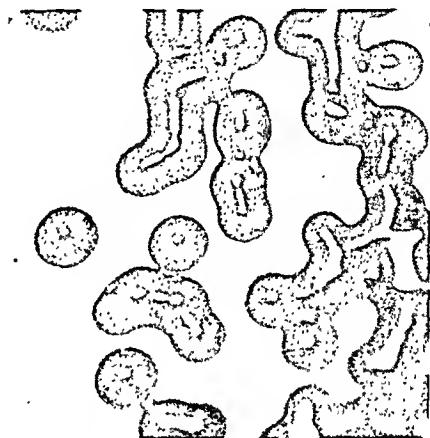


Fig. 2.

Strain 567m, 7 days at room temperature on bromthymol blue lactose agar. 5 X.

strains contained the same M-antigen, and this was also present in the strains 567S(m) and 567R(m)L + — but not in the other S or R strains.

The small translucent blue colony (no. 5 above) could be maintained in a reasonably pure form by selection of small blue colonies which did not contain papillas or daughter colonies, for subculture.

Acid production from lactose in fluid medium.

The acidity produced during lactose fermentation was studied by inoculating several tubes of 1 % lactose in unbuffered meat infusion broth with a number of strains. The tubes contained 10 ml of the medium and all tubes were inoculated with 1 drop of suspensions containing about 1000 million organisms per ml.

Table 1 shows the results of a comparison between the lactose-fermenting and lactose-negative strains of 411 and 700₁.

All lactose-fermenting strains seem to produce about the same acidity, and the strains 700₁ML— and 700₁SL— only lag slightly behind during the first 24 hours, showing that a sufficient number

Table 1.

Determination of pH in 1 % lactose broth by glass electrode. Comparison of lactose-negative (L—) and lactose-fermenting (L+) strains.

Strain	24 h.	2 d.	3 d.	4 d.	6 d.	9 d
700 ₁ ML—	6.78	5.69	5.54	5.37	5.18	5.03
700 ₁ ML+	5.88	5.82	5.58	5.45	5.21	5.03
700 ₁ SL—	6.50	6.09	5.72	5.44	5.28	5.08
700 ₁ SL+	6.17	5.82	5.53	5.32	5.11	5.03
700 ₁ RL—	6.62	6.63	6.82	6.49	5.37	5.08
700 ₁ RL+	5.92	5.72	5.65	5.38	5.14	5.01
400 ML—	6.72	6.93	6.98	5.56	5.74	5.52
411 ML+	5.81	5.57	5.38	5.37	5.41	5.38
411 SL—	6.83	6.75	6.42	6.22	6.01	5.24
411 SL+	5.65	5.62	5.27	5.17	5.14	5.06

of mutants has appeared to cause a rapid fermentation. The strains 411ML— and 411SL— on the other hand, apparently cause a slow gradual change of the acidity at least during the first few days. The interpretation of these results is difficult, however, due to the disturbing influence of the mutations. It seems however, as if mutable strains may differ in their primary action on lactose. Whereas some strains appear to be entirely inactive until mutants appear, others may possibly be able to effect a very slow fermentation. Similar differences have been observed by Kristensen (3).

Table 2.

pH determinations in lactose broth. Comparison of pale yellow (S₁) and dark yellow (S₂) strains, and of green (G) and yellow (Y) strains.

Strain	24 h	2 days	3 2ays	4 days	6 days	9 days
Bj ₁ S ₁	5.33	5.28	5.10	5.03	5.02	4.95
Bj ₁ S ₂	5.87	5.64	5.48	5.33	5.17	5.03
248 S ₁	5.61	5.57	5.42	5.36	5.24	5.19
248 S ₂	5.07	5.01	4.93	4.92	4.92	4.86
329 MG	6.59	5.72	5.81	6.15	5.93	6.03
329 MY	6.59	5.68	5.80	5.95	5.94	6.00

Table 2 shows comparison between the pale yellow and strongly yellow (S₁ and S₂) strains. There does not appear to be any significant difference between the two types, and it may be reasonable to assume that the color difference is not due to different modes of attacking lactose, but rather to some chemical difference which makes the dark yellow form adsorb the indicator dye more strongly than the pale yellow one.

Table 3.

pH determinations in lactose broth. Comparison of different mutants of strain 567.

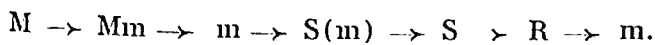
Strain	24 h.	2 days	3 days	4 days	6 days	9 days	10 days
567 S (m)	5.60	5.25	5.07	5.04	5.04	5.00	
567 S	5.53	5.27	5.15	5.07	5.20	4.98	
567 RL—	6.24	5.58	5.95	6.42	7.05	6.00	8.42
567 RL+—(a)	5.91	5.88	5.79	5.96	6.77	8.05	8.11
567 RL+—(b)	5.99	5.77		7.51	7.56		9.17
567 RL+—(c)	5.71	5.58		5.53	6.97		5.52
567 RL+—(d)	5.93	5.92		6.47	6.32		8.33
567 RL+—(e)	5.70	5.73		5.30	8.38		7.30
567 RL+	5.48	5.32		5.26	5.25		5.33

L+: rapid lactose fermenters. L—: lactose negative on plates. L+—: acidification of plates followed by alcalinization. Strains (a), (b), (c) etc. are different strains of the same type, originating from different colonies.

Table 3 records the results obtained with different mutants of strain 567. Whereas all the yellow colonies behave in the same manner, those which were blue or which turned blue after initial acidification showed a very irregular behaviour, with apparently little agreement between different culture tubes. These results seem to be difficult to explain. It was thought that these strains might perhaps ferment carbohydrates in a similar manner as *Aerobacter* strains, but as they all showed a negative Voges-Proskauer reaction, this assumption could not be substantiated.

Discussion.

Whereas dissociation from M to S in most cases seemed to be a very simple process, which is reconcilable with the assumption that it is due to the mutation of a single gene, the behaviour of strain 567 shows that the process may be much more complicated and pass through several steps. It is not suggested that these steps form a continuous series. It seems possible that the process may be more irregular than that, still the changes that actually were observed can be illustrated by the following series:



The question then arises whether the mucoid forms at either end of this series are equivalent. In favor of their different nature it should be mentioned that their consistency was entirely different, those at the beginning of the series being very viscous and stringy, whereas those at the other end appeared to be much less viscous.

Still it was shown that the M-antigen was the same. The difference might possibly be explained by assuming that the first steps in the series are S-forms, or even capsular forms in the Sense of Kauffmann, with an extra envelope of M-antigen (the MO or MKO forms suggested by Kauffmann (2)), whereas the last steps might be R-forms with an envelope of M-antigen (Kauffmann's MR). If this is so it may not always be entirely correct to visualize the dissociation from M through S to R as a gradual degradation of the organisms. If it can be shown in other organisms that M-forms can be produced from both S-forms and from R-forms, it would necessitate reconsideration of some of our ideas about dissociation. It should be mentioned that Moller (4) also obtained M-forms from R-forms, and the author has also previously seen such phenomena, which were difficult to explain otherwise (partly unpublished results).

In the strain 567 the apparent complexity of the dissociation may perhaps partly be due to the fact that several different antigens were being changed at the same time. Some of the serological results reported in the following paper strongly suggest that this strain also had lost another antigen than the M-antigen on the way from M to S. Thus the different behaviour of the different strains might partly be due to the fact that some strains were simple MO forms, whereas 567 and 173 were MKO forms. It would require a great deal of additional work to get a really satisfactory explanation of the observations. It seems questionable that the production of several different mucoid colonies and colonies with slime wall production, all apparently containing different quantities of the same M-antigen, would be reconcileable with the hypothesis that these variations were due to mutation of a single gene. It seems that some more complicated mechanism would have to be postulated.

The studies of the lactose fermentation, on the whole, gave results that are in good agreement with previously known facts. The early observation of Baerthlein (1) that mutable strains can be isolated from apparently normal strains of *E. coli* was confirmed. It seems probable that this type of variations or mutation is very common in this species, and there may be no very important difference between mutable strains and lactose-fermenting strains. It is apparent that the mutation rates in both directions may be highly different in different strains, and this fact may be one of the reasons why certain strains show a greater tendency to appear as mutable strains than others. One may also assume that under certain, as yet unknown circumstances, a selection pressure, favorable to the lactose-negative type, would be set up. In such a manner one might explain the frequent occurrence of mutable strains — even with high mutation rates — in certain pathological conditions, such as chronic urinary infections.

The reason why these different kinds of variation have been treated under the same heading was that it was at first thought that these

variations might be connected in some manner. Certain observations might suggest that this was so, thus the slime wall production of the lactose fermenting mutant 700₁RL + and the production of lactose-fermenting mucoid strains from the lactose negative strain 567RL —. Similar observations have been made in previous experiments. Thus in one case a smooth strain, which did not ferment lactose nor produce gas, after passage through a fluid medium for 24 hours, split off a mucoid, lactose-fermenting gas-producing strain.

In most cases, however, mutation with respect to lactose fermentation and to colony type appear to occur independently, and the few exceptions might easily be explained as coincidental.

Some irregularities in the lactose fermentation of some strains were observed. Thus some apparent R-forms of strain 567 had a tendency to alcalinize the media, both solid and fluid, after initial acidification. The reason for this behaviour was not found. It seems likely that these strains either had lost part of their ability of breaking down the lactose or that they had acquired the faculty of further breakdown of the acid fermentation products. The irregularity of the results obtained in fluid media suggests that mutation might have occurred in some of the tubes, although these strains seemed to be stable on solid media.

The slight color differences between different colonies, both of M and of S-forms, which appeared as a difference in the shade of yellow on bromthymol blue lactose agar, did not appear to be due to differences in the acidity. The slight differences in opacity, and in the size of the colonies in the case of M-strains, suggests that this phenomenon might be due to some chemical change of the organisms which altered the affinity of the bacteria to the indicator dye. This type of variation seemed to produce more stable variants in the S-forms than in the M-forms. It is not certain that the variation is the same in both colony phases, and the significance of the phenomenon, if any, is unknown. In one case which was studied very carefully (248S₁ and 248S₂) it was found that the two variants were serologically identical both in agglutinin titrations and in quantitative agglutinin determinations.

Summary.

Several mucoid strains of *Escherichia coli* were studied with respect to dissociation and mutative lactose-fermentation.

In most cases dissociation from M to S occurs in one step, but one strain produced several intermediate steps between M and S which seemed to produce decreasing quantities of the M-antigen. Spontaneous mutation from R to M was observed. These M-forms appeared to differ from the original ones, and it is suggested that the latter might be MKO or MO forms and the former MR forms.

The same strain split off lactose-negative mutable strains in old broth cultures. Other mutants produced an alkaline reaction in lactose-media after initial acidification.

Some M-strains produced two types of colonies which differed from each other in size, translucency and color on bromthymol blue lactose agar. The two types were unstable. Some S-strains also produced two types of colonies, which appeared to be more stable and which differed from each other in the shade of yellow color of colonies on the same medium. No constant difference in the acidity produced in lactose media by such variants could be demonstrated. The significance of the findings is discussed.

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STUDIES IN MUCOID ESCHERICHIA COLI II. SPECIFICITY OF THE MUCOID ANTIGEN (M-ANTIGEN)

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It was reported in a previous paper (7) that several mucoid strains of *Escherichia coli*, collected at random from different patients, appeared to have closely related M-antigens (the term M-antigen is used rather than capsular antigen to avoid confusion with the capsular antigens or K-antigens studied by Kauffmann, Vahlne and others (9, 11, 15, 16)). This paper reports some additional studies, undertaken in order to gain more information about these cross-reacting antigens.

Material.

Most of the strains were the same as described in previous papers (7, 8). Their designations and general appearance are listed below. The origin of each strain is indicated by the number of the pathological specimen from which it was isolated. Different strains from the same specimen are distinguished by subnumerals after this number, and different colony-phases of the same strain by the letters M (large mucoid colony), m (small mucoid), S and R.

- I. Strongly mucoid strains with large, domed, translucent colonies:
 - A. Colonies with the consistency of thin agar jelly or very slightly stringy. 248 M, 700₁M, 411 M, 41 M, 241 M, 60 M, B₁1M.
 - B. Viscous, very stringy colonies: 329 M.
 - C. Extremely viscous, tough colonies: 567 M, 173 M (from the same patient with an interval of several months).
- II. Small, mucoid, semiopaque colonies of soft watery or stringy consistency: 700₂m, B₂2m, 567 m.
- III. S-forms of the same strains, either isolated from the original specimen or from broth cultures of the M-forms: 248 S, 411 S, 700₁S, 700₂S, 41 S, 60 S, B₁1S, 329 S, 173 S.
- IV. Smooth or slightly irregular, dry colonies with most of the properties of R-forms, isolated from the original specimen or from old broth cultures: 700₁R, 567 R, 173 R.

Both lactose-fermenting and lactose-negative mutants of several strains were studied, likewise two different mutants or variants of several strains which differed from each other only in the colour of the colonies on brom-thymol blue lactose agar (orange and pale yellow respectively). As all these mutants of the same strain behaved identically in serological reactions, no further mention will be made of them.

Methods.

Immunization. Rabbits were given intravenous injections of heated organisms (2 h at 100 C, for production of O-sera), or formalin-killed suspensions (for production of anti-M or anti-S immune sera). As some antigens were quite toxic, dosage and intervals had to be varied according to the tolerance of the rabbits. 6 to 8 injections usually sufficed for production of anti-O or anti-S immune Sera, but production of antibodies against the M-antigen was slow and irregular, and immunization had to be continued for 4 weeks and more. Even then most animals failed to produce satisfactory immune sera, and in such cases immunization was continued with living antigens. Occasionally this had the desired effect, but frequently the rabbits succumbed to the toxic effect of living antigens. Anti-M immune sera always showed very low agglutinin titers, rarely above 1:10 or 1:20, and some were active only in concentrated form.

Agglutination tests were partly carried out as slide tests, partly in tubes with twofold dilutions of serum and equal volumes of antigen suspensions containing about 2000 millions organisms per ml. The latter tests were incubated at 37 C, and read after 2, 4 and 20 hours. Precipitation tests were carried out by layering antigen dilutions above equal volumes of serum (0.1 to 0.2 ml). The tests were read as ring tests, after mixing and incubation at room temperature for 1 hour, and finally after 20 hours at 4 C. Tubes which appeared to be negative were centrifuged at 2000 r. p. m for $\frac{1}{2}$ hour before final readings.

Complement fixation tests: twofold dilutions of inactivated serum, complement (2 100 % units) and antigen (about 1000 million/ml), all in 0.2 ml volumes were mixed and incubated at 4 C for 18–20 hours. 0.4 ml of 1.5 % sensitized sheep erythrocytes were added, and the tests read after 15–20 minutes at 37 C.

Absorptions: immune sera were mixed with washed and packed organisms from 6 agar slants per ml, a quantity found sufficient to remove all agglutinins against S or O antigens, left at 4 C over night and centrifuged.

Quantitative precipitin determinations: The method of *Heidelberger* and *Kendall* described in (5) and numerous others papers, was used. Antigen doses were determined by precipitin tests in 0.1 or 0.15 ml volumes of serum with different antigen dilutions, followed by tests for excess antigen or antibody in the supernates. Whenever possible it was attempted to do at least one set of determinations in the region of slight antigen excess.

Isolation of polysaccharide antigens. In some experiments the method used by *Heidelberger*, *Goebel* and *Avery* (6) for isolation of *Klebsiella* polysaccharides, was used. Organisms from 30 to 50 agar plates, grown at 30 C for 48 hours, were suspended in saline and treated according to the directions given in (6), except that the organisms were not killed by heat. The solutions remained very viscous throughout the process, but after desiccation of the product over P_2O_5 , a considerable portion of the substance had become insoluble, and the resulting solution, which contained abundant alcohol-precipitable substance, was no longer viscous. Serological tests indicated that it was the M-antigen that had become insoluble, and that only some other polysaccharide (probably from the somatic antigen) and some impurities remained.

As this method apparently failed, and also resulted in very impure products, a simpler method of isolating the M-antigen was tried. Organisms from 3 lactose agar plates, cultivated at 30 C for 48 hours, were suspended in about 50 ml saline, and centrifuged at high speed (about 4000 r. p. m.) until the organisms had sedimented (usually several hours were necessary). The supernate, which was very viscous and slightly opalescent, apparently contained a major portion of the M-antigen. It was diluted to about 200 to 250 ml to reduce viscosity and passed through Berkefeld candles.

In one case (567 M) the organisms did not come down on centrifugation, and the whole suspension had to be passed through the filter. The filtrates were treated with sodium acetate and 2 volumes of ethanol, and the resulting precipitate was centrifuged out and the supernate drained carefully off. Finally the precipitates were dissolved in saline. The strongly mucoid strains yielded light brownish, fibrous precipitates, slightly mucoid strains of S-strains only very small quantities of flocculent precipitate. The former solutions were very viscous, even in very dilute solution (0.1 % or less), whereas the latter were not appreciably more viscous than the solvent. Antigen solutions as well as immune sera were preserved with 0.01 % of merthiolate and stored at 4 C.

For study of the capsules the india ink method of Butt, Bonyng and Joyce (1) was used. Whereas this method permits a very good estimate of the size of the capsules, it does not draw a sharp line between capsulated and non-encapsulated organisms, as even S or R organisms are surrounded by narrow unstained halos.

Results.

Agglutination tests with S-antigens in anti-M, anti-S or anti-O immune sera (table 1) showed that the strains belong to several (at least 4) different O-groups. Only two strains (411 and 700₁) were shown to have the same O-antigen. Results with heated O-antigens in the same sera gave identical results, showing that the strains were O-forms and not capsular forms in the sense of Kauffmann (2). Strains 700₁ and 700₂ from the same specimen were serologically different and probably are different strains. 700₁R, also from the same specimen was unstable in saline and its O-antigen could not be determined, but according to other results (mutation rate with respect to lactose fermentation, and complement fixing capacity) it was thought to be an R-mutant of 700₁M, or an intermediate RS-form.

Results of agglutination tests with M-organisms are presented in table 2. Some strains (700₁M and 411M) were completely inagglutinable except in high concentrations of anti-M antibodies. Others (248M) frequently showed a late, slight partial agglutination nearly to the same titer as S-antigens, regardless of whether anti-M or anti-S sera were used. Others again (567M, 173M, 567m) are agglutinated to high titer by homologous anti-M serum, but to low titer or not at all in anti-S serum. These differences were unexpected in view of the antigenic similarities of the M-antigens, but may possibly be explained as due to structural differences in the different colonies (see later).

In complement fixation tests (table 3) M-strains react to the same

Table 1.

Agglutination reactions of S-organisms in different immune sera.
Final readings after 20 hours at 37 C.

Antigen	Immune sera					
	248 M	700 ₁ M	567 M	567 m	248 S	567 S
248 S	10240	0	0	0	10240	0
567 S	160	160	10240	5120	40	2560
173 S	160	40	10240	2560	40	2560
60 S	0	0	40	20	0	20
700 ₂ S	40	40	40	20	0	0
700 ₁ S	40	5120	20	20	0	20
411 S	40	5120	40	20	0	20
41 S	320	80	160	160	—	—
Bj ₁ S	20	20	20	0	20	0
329 S	80	40	40	40	—	—

O: no reaction with lowest dilution used (1:20 or 1:40).

—: reaction not done.

Tests with O-antigens (heated) of 248 S, 700₁S, 60 S and 567 S gave the same results as with S-antigens, likewise tests of most antigens in several anti-O sera.

Table 2.

Agglutination reactions in test tubes with M-organisms. Readings after 20 hours at 37 C.

Antigen	Immune sera				
	248 M, 1	700 ₁ M, 2	567 M	567 m	567 S
248 M	40—5120*)	0	0	0	0
567 M	0	0	5120	10240	0
567 m	0	0	20480	10240	320
60 M	0	0	0	0	0
700 M	0	0	0		
411 M	0	0	0		
41 M	0	0	0		
329 M	0	0	0		
Bj ₁ M	0	0	0		
700 ₂ m	0	0	0		
567 S			20480	10240	2560

*) These reactions were variable with different batches of antigen, but always very slow (only after 20 h), partial and finely granular. Similar reactions were obtained with several anti-S and anti-O sera.

Table 3.

Complement fixation reactions with the M-strains and several S- and R-strains.

Antigen	Immune sera		
	248 M, 1	700 M, 2	567 M
248M	1536	192+	384+
248S	1536		
567M	192---	192+	1536
567S			1536
567m		192+	1536
173M		192+	1536
60M	192	192+	384+
700M	96+	768+	768+
700S	192	768+	
700R		384+	
411M	192	768+	768-
329M		384+	768
41M		192	192
Bj ₁ M	192—	192+	192+
700 ₂ m		384-	768

Several other S-strains, not included in the table gave the same results as the homologous M-strains. Tests in several other sera gave similar results.

titers as S-organisms, regardless of agglutinability. Cross-reactions are more marked than in the agglutination tests, and are more uniform, suggesting that they may be determined by some common antigen which plays little role in agglutination. The results are not very specific, although slightly more so than in some anti-Klebsiella immune sera (7). The somewhat stronger homologous reactions are probably due to the specific O-antigens, whereas Klebsiella strains contain only species specific somatic antigens in addition to the type-specific capsular polysaccharides (2, 3, 15). (However, see Kauffmann (2)).

Slide agglutination tests with M-organisms in anti-M sera gave the results shown in table 4. In sufficiently potent immune sera (e.g. 248M, 1 or 700₁M, 2) the strongly mucoid strains gave very characteristic reactions with voluminous, jelly-like or fibrous precipitates. The small mucoid type of colony either gave a similar reaction (567m) or a flocculent reaction (700₂m and Bj₂m). No cross-reactions were found between this M-antigen and a number of immune sera against other mucoid organisms (different Klebsiella types, pneumococcus types and several other mucoid organisms). Immune sera that had been exhausted of all agglutinations by absorption with the homologous S-organisms gave just the same reactions with all mucoid strains, showing that the M-antigen is only present in mucoid strains.

Table 4.

Slide agglutination tests with mucoid strains in unabsorbed and absorbed anti-M immune sera.

Antigen	Immune sera					
	Unabsorbed			Absorbed with homologous S-strain		
	248 M. 1	700 ₁ M. 2	567 M	700 ₁ M. 2	567 M	248 M. 2
248M	+++	++	++	++	++	+++
700 ₁ M	+++	+++	+++	+++	++	+++
567M	+++	+++	+++	+++	+++	+++
411M	+++	+++	+++	+++	+++	+++
Bj ₁ M	+++	++	+	++	+	+++
60M	+++	+	+	++	+	+++
173M	+++	+++	+++	+++	+++	+++
41M	+++	+++	+	+++	+	+++
329M	+++	+++	+++	+++	+++	+++
241M						+++
700 ₂ m	+	++	+	++	++	+
Bj ₂ m	+	+	++	++	+	++
567m	+++	+++	+++	++	++	+++
248S						0
700 ₁ S				0		
567S					0	

+++ : compact precipitate, ++ : smaller precipitate in threads or coarse flakes, + : small floccules.

Slide tests in anti-S sera were negative, likewise tests with immune sera against *Klebsiella* types A, B, C, D, AE, several pneumococcus types and several other mucoid gram-negative rods.

Some of the weaker immune sera seemed to bring out differences between some of the strains. These differences, however, probably do not indicate different antigenic specificity of the M-antigens, but rather differences in the availability of this antigen in different strains.

Suspensions of colonies which contained mostly extra-cellular M-antigen, probably produce more finely dispersed solutions than colonies which have the M-antigen arranged in large, well defined capsules. In the former, the reaction may, in all essentials, behave as a precipitation reaction, sensitive to antigen excess, whereas in the latter the reaction may mainly occur at the surface of the capsules and behave as an agglutination. Studies of the reactivity of M-antigens, prepared from Berkefeld filtrates of different strains, and of capsule-preparations seemed to give evidence in support of this assumption.

Some of the properties of purified antigen solutions are shown in table 5. It seems obvious that the antigens prepared by the trypsin digestion method (6) differ from those prepared from Berkefeld filtrates.

In order to get a rough estimate of the quantity of material contained in the Berkefeld filtrates, 5 ml portions of the two most concentrated solutions were acidified with acetic acid and precipitated with alcohol. The precipitates were centrifuged out and dried to constant weight at 100 C. It is not believed that these products were free from ash or other impurities, so the weights calculated on this basis

Table 5.

Properties of some purified antigen solution, prepared by the trypsin digestion method of Heidelberger, Goebel and Avery (T), or from Berkefeld filtrates (B).

Preparation	Yield G	Volume of solution	Biuret test	Molisch test	N, % of weight	Viscosity	Serological reactivity
248 M (T)	(0.875)**	100 ml	—	+++	—	—	10 ⁻⁵ *
567 M (T)	(0.481)**	100 ml	—	+++	(2.1 %)**	—	10 ⁻⁵ *
567 m (T)	(0.239)**	25 ml	—	+++	(1.1 %)**	—	10 ^{-4.4} *
248 M (B)	0.0768	30 ml	—	+++	0.8 %	+++	2.6 x 10 ⁻⁸
567 M (B)	—	50 ml	—	+++	—	++	10 ^{-4.7} *
B ₁₁ M (B)	0.059	25 ml	—	+++	0.5 %	+++	2.4 x 10 ⁻⁸
700 ₂ m (B)	—	2 ml	—	++	—	—	1/20*
567 m (B)	—	10 ml	—	+++	—	+	10 ⁻³ *

*) When the weight of the product was unknown, the serological reactivity has been expressed by the highest dilution that still produced a precipitate, assuming the value of 1 for the weight of the product. Actually the weights were far smaller than 1 g (from about 0.1 to 0.5 g for the trypsin-antigens and from a few mg to less than a mg. for the Berkefeld filtrates). Thus, these figures would have to be divided by factors of 2 to several thousand to get the actual dilutions.

**) A portion of these fractions, tentatively estimated at about 50 %, remained insoluble. Thus the figures for the yields are too high and the N-percentages too low.

All Berkefeld filtrates were produced from the same number of lactose agar plates (3), and the trypsin-fractions from 30 to 50 agar plates.

All antigen solutions gave positive tests for reducing sugar after acid-hydrolysis only.

probably are too high. The trypsin-fractions apparently contain considerable quantities of nitrogen, whether due to impurities or to some constituent of the polysaccharides is unknown. The small quantity of N found in Berkefeld-filtrates is believed to indicate some impurity. Both types of preparation are serologically reactive, and give precipitates with immune sera in high dilution. The M-antigen is at least equally as reactive as other bacterial polysaccharides, as it gives a slight trace of precipitate even diluted 1 : 40 million.

Results of precipitation tests with different antigens (table 6) clearly show that the trypsin-antigens and the Berkefeld-antigens are different. The former give practically only reactions with homologous

Table 6.

Titration of the reactivities of the purified antigens by the precipitation reaction. 10-fold antigen dilutions with equal volume (0.1–0.2 ml) of unabsorbed or absorbed serum.

Serum	Antigen	Antigen dilutions					
		undil. *)	1:10	1:100	1:1000	1:10000	1:100000
248 M, 2	248 M (T)	++	++++	++	tr	0	0
- -	567 M (T)	tr	tr	0	0	0	0
567 M	248 M (T)	tr	tr	tr	0	0	0
- -	567 M (T)	+++	+++**)	+++	+	0	0
- -	567 m (T)	+++	+++**)	+++	+	0	0
567 S	567 M (T)	0	0	0	0	0	0
- -							
- -	567 m (T)	0	0	0	0	0	0
700 ₁ M, 2***)	248 M (B)	0	+++**)	+++	++	+	tr
- - -	567 M (B)	+++**)	++	++	tr	0	0
- - -	Bj ₁ M (B)	0	+++)	+++	++	+	tr
- - -	700 ₂ m (B)	++	+	0	0	0	0
- - -	567 m (B)	++	+	tr	0	0	0
- - -	248 S (B)	0	0	0	0	0	0
567 M***)	248 M (B)	0	+	+++**)	+	+	tr
- -	567 M (B)	++	+++**)	+	tr	0	0

*) The original solutions, shown in table 5, column 3, were considered as undiluted antigen.

**) These antigen doses gave slight excess of antigen in supernates.

***) These sera were absorbed with the homologous S-organisms.

immune sera, and may be a part of the O-antigen, whereas the latter react equally well in all anti-M sera, even after absorption of all somatic agglutinins.

Quantitative precipitin determinations with trypsin antigens (table 7) show that this must be a major antigen, as it removes a considerable quantity of antibody from the serum. Tests of the supernates with different bacterial antigens (table 8) show that a large fraction of the somatic agglutinins has been removed, whereas the anti-M antibody apparently is unaffected. Thus these antigens at most contained traces of M-antigen. The trypsin-antigens prepared from the strains 567M and 567m seem to be equivalent, but differ from that prepared from 248M.

Similar tests with M-antigens (table 9) apparently give conclusive evidence that, at least, these two preparation are serologically identical. It appears from the qualitative tests that all the mucoid strains contain the same antigen, although in greatly different quantities. Thus it appears that the difference between the large and the small mucoid

Table 7.

Quantitative precipitin determinations by the method of Heidelberger, Kendall and Soo Hoo, with antigens prepared by the trypsin digestion method of Heidelberger, Goebel and Avery.

Immune serum	Mg antibody N*) precipitated from 1 ml serum				
	Antigen 567 M (T), ml			Antigen 567 m (T), ml	
	0.03	0.09	0.13	0.015	
567 M	0.12	0.19	0.25 **)	0.24 **)	
567 m	0.06			0.18 **)	

*) The N-content of the antigen doses used was negligible and could be disregarded.

**) The supernates from these tests showed slight antigen excess. The antigen doses are given in ml of the solution in table 5, column 4.

Table 8.

Agglutinin tests in supernates from precipitin determinations (table 7).

Serum	Absorbed with	Slide agglutination with				Test tube agglutinations with		
		567 M	567 m	567 S		567 M	567 m	567 S
567 M	567 M, 0.03 ml	++	++++	++++		640*)	640	640
- -	567 M, 0.06 ml	++	++++	++++				
- -	567 M, 0.13 ml	++	++	++				
- -	567 m, 0.015 ml	++	++	++		0	320	640
567 m	567 M, 0.03 ml	- **)	++	++		0	1280	640
- -	567 m, 0.015 ml	- **)	++	++		0	640	640

*) Slight partial agglutination only.

**) This serum also failed to agglutinate this antigen before absorption. The agglutinin titers of these sera before absorption are shown in tables 1 and 2.

Table 9.

Quantitative precipitin determinations with two different M-antigens produced from Berkefeld filtrates, in serum 248 M,2, after absorption with strain 248 S. Results calculated to 1 ml of serum (actual quantities used 2/3 ml).

Antigen 248 M (B), mg	Mg antibody N*) per ml serum	Antigen Bj ₁ M(B) mg	Mg antibody N*) per ml serum
0.38 **)	0.22	0.35 **)	0.21
0.19	0.19	0.17	0.17
0.095	0.17	0.085	0.17

*) As the N-content of the antigen was negligible (0.003 and 0.0018 mg respectively in the highest doses used), the total N was considered as antibody N.

**) These doses of antigen left slight antigen excess in supernates. The results are averages of duplicate analyses (as the results in table 7).

colony may be quantitative rather than qualitative, and that the mucoid growth in both cases is due to production of the same antigen.

Studies of the capsules of the different strains gave some results that might be thought to explain some of the differences found between different strains. 3 strains (700₁M, 411M and 329M) had very large, well defined capsules. Those of strain 411M (fig. 1) were particularly large. The strains 567M, 248M, 60M, 173M, 41M and B_j₁M showed more heterogeneous pictures. Some rods were surrounded by fairly large capsules, others by capsular spaces which were no larger than those seen in S or R strains. Thus these strains might appear

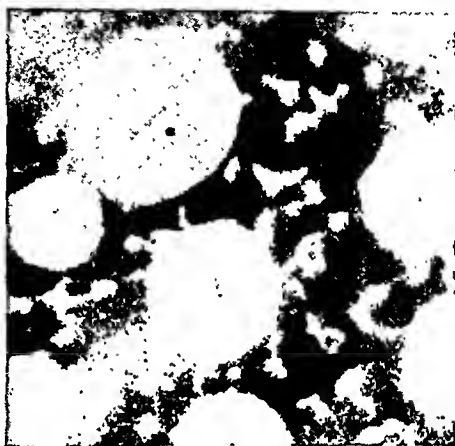


Fig. 4.

Strain 411M. India ink preparation from lactose agar culture. Counter-stained with gentian violet. $\times 1200$.

to be mixtures of encapsulated and non-encapsulated organisms, embedded in extracellular M-antigen, which was clearly visible between the cells in many films. The slightly mucoid strains, 700₂m, 567m and B_j₂m were surrounded only by very narrow capsular spaces, such as found in S-strains.

Thus there are apparent differences both in the quantity and the arrangement of the M-antigen. In some strains it is mainly arranged in large capsules, in others mainly as an extracellular excretion. The former strains were most consistently negative in agglutination tests with homologous anti-S sera, whereas one of the latter (248M) showed a slight, slow agglutination to high titer. It is probable that only the non-encapsulated rods in these colonies were agglutinated, and that the reaction was delayed by the viscosity of the suspension. The strains with the greatest tendency to give negative slide agglutination tests with weak anti-M sera were found to be those which had the largest quantities of extracellular M-antigen.

The strain 567M was agglutinated to high titer by the homologous serum.

The explanation might be similar as with the strain 248M, but the agglutination was complete in all dilutions, and the strain also differed in other respects from 248M and the others, thus in its colony dissolution, and it seems likely that other antigenic differences between this strain and the others played a role.

Discussion.

The results show that 13 mucoid strains of *Escherichia coli*, isolated from 10 different patients, and apparently representing 12 different strains, have serologically indistinguishable, probably identical, M-antigens, present in the mucoid phase only. This suggests that this M-antigen must be very common in mucoid *E. coli*. As the collection of strains is small, more general conclusions are not justified, and further studies of larger numbers of strains will be necessary to decide the general validity of the results. There was no correspondence between the M-antigen and the O-antigens as the strains belonged to at least 4 different O-groups, and only two strains were actually shown to have the same O-antigen.

The M-antigen appears to be a complex polysaccharide, similar to other bacterial capsular polysaccharides. It would seem to be interesting to establish the relationship, if any, between this antigen and the dextrans and levans studied by Hehre and others (4). The M-antigen does not seem to be readily soluble, as shown by the comparative difficulties in preparing even saline suspensions, and by the fact that desiccated preparations became insoluble. The viscosity is very high, even in very dilute solution (0.1 % and less). The antigen can be separated from the other bacterial antigens by centrifugation and/or filtration. The antigenic effect on rabbits seems to be poor and irregular, as only a few of the rabbits used produced satisfactory immune sera, and many produced no detectable anti-M at all.

In precipitation tests with immune sera the M-antigen is reactive in extremely high dilution (about 1 to 40 million). This reactivity is at least comparable to that of other bacterial polysaccharides. The high reactivity may possibly to some extent be due to the very gentle methods used in the preparation.

A similar antigen could also be detected in filtrates of slightly mucoid cultures, but in much smaller quantities (probably about 10^{-4} to 10^{-5} times as much in strain 100_m and 567_m), as judged by the titer of the solutions, prepared from the same quantities of culture.

Apparent differences in the reactivity of different strains in weak immune sera are believed to be due to antigen excess inhibition phenomena, caused by finely dispersed solution of free M-antigen in the case of strains that contain much extracellular M-antigen. With

such solutions a precipitation reaction would be obtained rather than agglutination. Solutions prepared from both types of strains were shown to be equally reactive in the precipitation reaction.

It seemed surprising that strains with the same M-antigen should produce colonies of such widely different size and consistency. In pneumococcus or *Klebsiella* the same sero-type usually produces the same type of colony. This discrepancy may partly be due to different arrangement of the M-antigen in individual strains (mainly in the form of capsules or mainly extracellularly), partly by quantitative differences (small mucoid and large mucoid colony respectively), but it seems probable that other antigenic differences may also play a role. Otherwise it would be difficult to explain the difference between the strains 248M (thin jelly) and 567M (very tough colony), both of which appeared to contain mainly extracellular M-antigen. It seems possible that strain 567M may have contained some additional capsular antigen (K-antigen), and represented the MKO-form suggested by Kauffmann (12).

The fact that strains with small mucoid colonies have the same M-antigen as strains with large colonies suggests that there may be several steps in the dissociation, or mutation, from M to S. This was shown clearly by the dissociation of strain 567M (8), which passed through several different steps (fig. 1 and 2 of preceding paper) in this process (large mucoid \rightarrow moderately large mucoid \rightarrow small mucoid \rightarrow non-mucoid with traces of M-antigen \rightarrow non-mucoid without M-antigen). Capsule-free, lactose-negative »mutabile« mutants of this strain, which apparently were rough in several important respects, showed reverse mutation to the small mucoid type, but these mutants, again, differed from the others in being much less viscous. It seems possible that the latter mutants may have represented either the MO or the MR type of Kauffmann.

This mode of dissociation by steps suggests an analogy with dissociation in pneumococci (14) or *Klebsiella* (2, 3, 15), both of which have been shown to produce intermediate forms between M and S.

One of the aims of this and previous studies was to search for evidence of type-transformation in vivo or »mutation dirigée« in the sense of Boivin (17)). The antigenic relationship between the strains 700₁M and 700₂m and between B_{j1}M and B_{j2}m, which otherwise seemed to be different strains, at first was thought to supply such evidence. While the possibility remains that these may actually have been cases of »type transformation«, the fact that this M-antigen is so common in *E. coli* weakens the hypothesis considerably. It seems to be equally probable that some kind of selection pressure in the milieu, that these strains were growing in, may have favored the establishment of mucoid mutants, produced independantly by both strains. If it should be found that this M-antigen is as common as suggested by this report, evidence of type-transformation would be very difficult to establish. The reason

why one of each pair of strains should appear as the small mucoid type, the other as the large one, is obscure.

The relationship between the M-antigen and the K-antigens of Kauffmann, Vahlne and others (9, 11, 16), has not been established. Vahlne found that his mucoid strains represented several different K-types. It seems possible that the presence of an M-antigen in addition to the K-antigen might have been missed due to the poor antigenicity of the former, but it is also possible that some apparent discrepancies may be due to differences in the numbers of strains studied.

A paper by Kauffmann (12), which appeared while this report being was prepared, also suggests the possibility of some further discrepancies between his results and those reported here and in previous papers (7, 8). The reason for such apparent discrepancies should perhaps be set aside until further studies have been made. One point should be mentioned however, namely the rather peculiar fact that all mucoid organisms isolated from the urinary tract in this laboratory could be classified as either *E. coli* or *Aerobacter* (probably preferably *Klebsiella* according to the opinion expressed by Kauffmann), whereas he finds *Klebsiella* strains very frequently in the urinary tract. It might seem as if the composition of the different materials must have differed a great deal.

Summary.

12 different strains of mucoid *Escherichia coli*, all from pathological processes, had the same M-antigen (mucoid antigen).

This antigen could be separated from the bacteria by centrifugation and/or filtration, and appeared to be a complex polysaccharide. It had a low and uncertain antigenic effect in rabbits, but was highly reactive in tests with immune sera (up to dilution $1 : 4 \times 10^7$). M-antigens from different strains appeared to have identical antigenic properties.

In spite of the common M-antigen the 12 strains differed rather widely with respect to size and consistency of the colonies, and the degree of capsulation of the rods.

Two pairs of strains from the same patients had the same M-antigen. The possible significance of this is discussed.

Whereas dissociation from M to S usually occurred in one step, one strain produced several intermediate colony types in this process, suggesting an analogy with dissociation in *Klebsiella* and in *Diplococcus pneumoniae*.

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A LITTLE EXPERIMENT WITH ACTH AND ANAPHYLACTIC SHOCK

By J. Ørskov.

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It has been shown in a little work (1) that sanoerysin, much used in Denmark against chronic rheumatism, if given to guinea-pigs sensitized to horse serum, can alter the anaphylactic shock so that a high percentage will survive as compared with the controls.

The very interesting effects arrived at recently with certain hormones against the same disease, made it tempting to examine if a similar effect would be found with these. As only few milligrams of ACTH (2) were available, the experimental possibilities were very limited. Guinea-pigs were sensitized, and 17 days later some of the animals were tested with intravenous injection (ear vein) of 0.2 ccm horse serum; all died inside few minutes, as in many earlier experiments.

Two of the guinea-pigs each injected with two milligrams i. p., tested 18 hours later, died promptly as the controls. Dr. Hamburger, chief of our Hormone Department, comforted me a little by saying that the effect might have disappeared, so the remaining 8 milligrams were used as follows:

10 sensitized animals were injected i. p. with 1/10 of the 8 milligrams dissolved in salt water, and then tested after augmenting time.

No. 1 tested 1 hour after the i. p. injection died astonishingly fast.

» 2	» 2 hours	»	»	»	»	survived
» 3	» 2	»	»	»	»	died
» 4	» 3	»	»	»	»	survived
» 5	» 3	»	»	»	»	survived
» 6	» 3	»	»	»	»	died
» 7	» 4	»	»	»	»	survived
» 8	» 4	»	»	»	»	died
» 9	» 5	»	»	»	»	survived
» 10	» 5	»	»	»	»	died

6 controls all died.

No. 5 had no clinical signs of shock, but all the others presented more or less pronounced anaphylactic shocks.

The experiments are few, hut as there can be little doubt as to the difference between the controls and the treated animals, maybe there is a hint in these results which may tempt other interested who have greater chances than we to get hold of the rare and costly substance.

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ERRATA.

Page 450, line 13: riboflavin, should be yeast.

Page 455, line 8: 1945, should be 1946.

Page 455, line 20: 186 and 201, should be 186—201.

Page 483, lines 20—21: by adding..... acid., should be by adermin
or pantothenic acid but in some experiments by adding yeast.

Page 473, line 3: the experiment period, should be the length of
experimental period.

Page 475, line 13: Phenol, should be Aniline.

Page 475, line 20: tumours, should be lesions.

Page 478, line 1: rat 1, should be rat 2.

Page 485, line 10: effect in, should be effect of.

Page 487, line 25: material C, should be series C.

Page 779, table 7: The tabulated number of P-negative parents is 66
instead of 88, of children 81 instead of 106. Consequently the
calculations of genefrequencies and distribution of offspring
into various classes are slightly altered, but the difference does
not affect the validity of the conclusions.

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